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13. ABSTRACT *(Maximum 200 Words)*

Exciting new findings in autoimmune disease and cancer have led to the realization that a large set of antigenic determinants of the self have not induced self-tolerance. These peptide determinants could provide target structures for autoimmune attack as well as antitumor immune responses. We hypothesized that vaccine strategies can be devised that specifically generate an immune response against breast ductal epithelial cells. Since the overwhelming majority of breast tumors arise in these cells, destroying these cells prior to the development of neoplasia will effectively prevent cancer. We are attempting to augment the immune response to the breast-specific antigen, HER-2/neu, which is expressed by mammary tissue in HER-2/neu transgenic mice prior to mammary tumor development, by enhancing the T cell response using selected vectors that may alter antigen processing and T cell activation, thereby influencing antigen-specific immunity. We have evaluated vaccinia constructs that express antigen alone or together with co-stimulatory molecules, to determine if immunity can be further enhanced. In our preliminary studies, we have found that this vaccinia approach is superior to vaccination with plasmid DNA. We are currently exploring additional methods of enhancing the potency of this vaccine approach, including testing a fusion protein that enhances the induction of helper T cell responses, and testing the vaccine in combination with other recently identified T cell activating signals such as CD40, OX40, and anti-CTLA-4. This study will provide a paradigm for novel vaccine approaches to breast cancer prevention.

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FOREWORD

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X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

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5. INTRODUCTION.

Breast cancer is a common problem, with an incidence of 182,000 new cases a year, and a mortality rate of 46,000 deaths per year. Overall, women in the United States have a 1 in 9-lifetime incidence of developing breast cancer (1-3). Because of its high incidence, breast cancer is a significant public health problem. Currently, most research is aimed at treating this disease. The time has come to focus efforts on prevention.

Recently, two breast cancer genes, BRCA1 and BRCA2, have been identified which influence susceptibility to breast cancer (4-6). Other genes associated with breast cancer susceptibility are likely to be identified in the future. It is estimated that approximately 5% of all patients who develop breast cancer, and 25% of patients who are diagnosed with breast cancer before age 30, will have a demonstrated susceptibility (7). With the current advances in molecular technology and the intensified effort to sequence the entire human genome, it is also very likely that other breast cancer susceptibility genes will be identified in the near future that are expressed by women at higher risk for breast cancer who do not have a strong family history. The identification of susceptibility genes should allow genetic screening for predisposition to this common deadly disease. Currently, the only option for prevention is surgical removal of the breast, which results in significant disfiguration and psychological trauma.

The goal of cancer prevention is to develop treatment modalities that specifically target the breast ductile tree, as well as preneoplasia and incipient tumor cells. Vaccine strategies that result in T cell activation directed against self proteins expressed by these cells have the potential to be effective treatment for this purpose because the diversity of the T cell repertoire allows for the recognition of greater than 10^6 distinct peptide determinants. In addition, it is well established that T cells recognize peptide fragments of cellular proteins bound to major histocompatibility molecules (MHC) on the surface of cells, and any cellular protein can be presented to T cells in this way.

Recent data has demonstrated that many antigenic determinants of the self do not induce self-tolerance (8-9). Instead, these peptide determinants can become antigenic targets for autoimmune attack, and therefore, may be potential targets for directing anti-tumor immune responses. Strong support of this concept comes from data demonstrating that the majority of human melanoma antigens that have been identified are normal tissue peptides that are overexpressed by the melanoma (10-16). Most importantly, melanoma patients receiving *in vitro* expanded tumor infiltrating lymphocytes (TIL) specific for a few of these antigens demonstrated clinical responses against metastases without evidence of significant tissue damage (17-18). Interestingly, *in vitro* cytotoxic T cells (CTL) can be generated against these same antigens from peripheral blood lymphocytes (PBL) of healthy donors without a history of autoimmunity (19). These data are consistent with the large body of evidence that both a critical density of MHC-peptide complexes on the target cell surface, as well as a critical set of co-stimulatory molecules,

are required to activate T cells. As these signals continue to be defined, it should be possible to manipulate them toward beneficial immune responses that result in preventative treatments, without causing unwanted toxicity. Our recent studies have provided strong evidence that the anti-tumor response can be considerably enhanced by recruiting subsets of lymphocytes that respond to tumor-specific antigens. Using murine models, we have demonstrated that the injection of tumor cells genetically engineered to produce local concentrations of cytokines, results in the activation of tumor-specific T cells capable of generating systemic anti-tumor immunity (20-22). In one study that compared over 10 cytokines for their ability to augment anti-tumor immunity, tumor cells genetically-altered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) produced the most potent systemic anti-tumor immunity (22). Analysis of the immune response generated has revealed that systemic immunity is dependent on both $CD4^+$ and $CD8^+$ T cells. Vaccination with GM-CSF secreting tumor cells has been shown to immunize mice against subsequent injections of parental tumor, as well as cure mice burdened with a relatively small amount of pre-established tumor. In addition, we have recently completed a phase I trial evaluating this approach in patients with advanced renal cell carcinoma (23). The results of this trial are extremely promising in that they demonstrate both clinical and immunologic activity. In addition, 3/3 patients receiving the bioactive dose of GM-CSF secreting vaccine cells demonstrated a DTH reaction against both autologous tumor cells, and autologous normal renal cells, yet no functional evidence of autoimmune damage was observed. This study provides further support for the existence of tissue-specific antigens that can serve as immune rejection targets in a second human tumor.

Two scientific questions need to be addressed prior to the design of vaccines that can prevent the development of breast cancer. The first requirement is the identification of antigenic targets expressed early by the pre-malignant cell that can be used for immunization. The second requirement is the identification of optimal methods for delivering these antigens to the immune system for priming anti-tissue immune responses.

Currently, the Her2/neu gene product, which is overexpressed by 25-40% of invasive malignancies (24), and by 56% of pure ductal carcinoma in situ (DCIS) (25), is the best antigen for targeting immune responses to prevent the development of breast cancer. There are two reasons for this. First, HER2/neu is selectively overexpressed by premalignant and malignant cells, but is also expressed by normal breast ductal tissue (26). Second, HER2/neu encodes a large protein (1255 amino acids) and therefore should contain a range of epitopes capable of binding to different MHC alleles. One major concern is that patients would be immunologically tolerant to self-proteins like HER2/neu and that immunity might be difficult to generate. However, Disis and colleagues recently described the identification of HER2/neu specific antibodies in 11 of 20 breast cancer patients studied. Interestingly, none of these patients had evidence of autoimmunity (27). A second study

demonstrated $CD8^+$ T cell responses against HER2/neu peptides from lymphocytes of a normal donor (28). These studies demonstrate the existence of T cell precursors that have the potential to recognize the endogenous HER2/neu gene product.

Muller and colleagues have developed a transgenic mouse that overexpresses the proto-oncogene Her2/neu under the MMTV promoter (29). The mice develop focal mammary tumors at approximately 6-8 months of age that metastasize with high frequency to lung. Expression of the proto-oncogene product in histopathologically normal mammary epithelium has been demonstrated prior to the development of these focal mammary tumors. There are at least two reasons why this model provides a unique opportunity to study antigen-based vaccine strategies for the prevention of naturally developing mammary tumors. 1) Because the Her2/neu product is overexpressed in the normal ductal epithelium of these mice prior to the development of mammary tumors, this model provides a unique opportunity to evaluate vaccine strategies for preventing the development of spontaneously arising primary tumors. 2) the product of the proto-oncogene HER2/neu is an excellent target for evaluating recombinant vaccine strategies for augmenting antigen-specific immunity, since it is overexpressed in several common human tumors.

The HER2/neu transgenic mouse model is being used to directly compare antigen-based vaccine strategies for the prevention of the development of mammary tumors. Specifically, we are evaluating vectors that express antigen alone or together with co-stimulatory molecules or cytokines to determine if this immunity can be further enhanced. The vectors that are being tested include: 1) plasmid vectors delivered intramuscularly or intradermally using a gene gun; 2) Vaccinia vectors carrying the HER-2/neu antigen alone, or a fusion protein consisting of HER-2/neu and the lysosomal targeting molecule (LAMP-1); 3) Listeria monocytogenes vectors. All three vaccine approaches were chosen based on previous experience demonstrating that these vectors can augment potent specific anti-tumor immunity against existing cancers (30-33).

One major concern with employing antigen-based vaccine approaches that target rejection antigens expressed by normal tissue prior to the development of malignancy, is that these antigens may go unrecognized by activated T cells at the levels at which they are expressed. Our collaborator, Dr. Sara Sukumar, has developed a gene therapy approach that can selectively ablate mammary epithelial cells in a rat model of breast cancer. Using viral vectors with high efficiency of infection (in particular, vaccinia and adenovirus vectors), her group has demonstrated the feasibility of preferentially targeting the epithelial cells by directly injecting the vectors into the primary mammary duct through the teat. Our working hypothesis is that we can increase access of activated T cells to normal ductal epithelial antigens by locally infusing vectors carrying a suicide gene such as the Herpes thymidine kinase (HTK) into the primary mammary duct, thereby rendering them susceptible to the toxic lethal effects of an antiviral drug such as

gancyclovir (34). Therefore, we are also testing the synergistic effects of combining this unique approach of accessing mammary epithelium with our recombinant vaccine strategies.

6. BODY

A. Hypothesis being tested (Assumptions).

This proposal is testing the hypothesis that the generation of autoimmunity against breast epithelial cell-specific antigens using recombinant vaccines will result in the destruction of the ductile tree, preneoplasias, and incipient tumor cells, thereby preventing breast cancer. The hypothesis is based on the fact that a significant number of human melanoma antigens that are the targets of T cells have recently been identified, and the majority of these antigens are normal tissue-specific antigens. These antigens are expressed by 40-60% of other patient's tumors, which provides strong support for the existence of common sets of antigens that can serve as targets for antigen-specific vaccine strategies. Most importantly, these antigens have served as tumor rejection antigens *in vivo*, resulting in clinical responses without functional evidence of destructive autoimmunity.

B. Experimental Methods.

Using the HER2/neu proto-oncogene transgenic mouse model of breast cancer, and the product of HER2/neu as a tissue-specific antigen for targeting immune responses, we are testing three recombinant vaccine strategies for the ability to: 1) activate antigen-specific immune responses and 2) prevent the development of breast cancer. The three strategies we are testing include: 1) GM-CSF secreting and HER-2/neu expressing 3T3 cells (3T3neuGM-CSF); 2) plasmid DNA containing HER-2/neu either alone or mixed with co-stimulatory molecule DNAs; 3) recombinant vaccinia containing HER-2/neu either alone or mixed with vaccinia containing co-stimulatory molecules. We had previously optimized all three strategies in the parental FVB/N mouse in which tolerance against HER-2/neu is not expected. From these experiments we determined that FVB/N mice were protected from a subcutaneous tumor challenge with a HER-2/neu+ tumor line following vaccination with either neu-encoding plasmid DNA, GM-CSF secreting irradiated neu-expressing cells, or vaccinia containing HER-2/neu. The addition of co-stimulatory molecules did not seem to significantly enhance the antitumor immune response generated. In the past year we have used these vaccine methods in the neu transgenic mouse model to determine their effectiveness in a setting where there is immunologic tolerance to HER-2/neu. Specifically, we have compared all three vaccine approaches for the ability to prevent both transplantable tumors and endogenously developing mammary tumors.

1. Evaluation of Recombinant DNA plasmid vectors.

Progress Report for the funding period 9/98 to 10/99

At the conclusion of year two, we planned to evaluate the efficacy of plasmid DNA vaccination against HER-2/neu in HER-2/neu transgenic mice. We had demonstrated previously that FVB/N mice were protected from a neu tumor (NT) challenge using both intramuscular and intradermal injections of plasmid DNA encoding HER-2/neu (in the pcDNA3 vector). In year three of this proposal, we extended these experiments to include vaccination of neu transgenic mice.

a. Procedure.

To evaluate the efficacy of plasmid DNA vaccination in HER-2/neu transgenic mice, we designed a series of experiments using DNA vaccination. We attempted to optimize the vaccine approach taking into account schedule of administration, boost, and priming multiple lymph node regions. We also compared HER-2/neu DNA alone with HER-2/neu given in combination with DNA encoding GM-CSF, B7-1 and B7-2. We also evaluated the approach together with the immune modulating antibody anti-CTLA-4. In the initial experiments, animals received 1 μg of neu-pcDNA3 given intradermally using the gene gun, followed by a subcutaneous NT challenge (5×10^4 NT cells) 14 days later. In order to enhance neu-specific immune responses in the transgenic mice, we modified this protocol in the following ways: 1) Animals received one, two, three, or four vaccinations prior to tumor challenge; 2) Multiple vaccinations were given at spatially distinct sites 3) Plasmid encoding GM-CSF, B7-1 or B7-2 was added to the vaccine (0.5 μg neu + 0.5 μg enhancer molecule); 4) Animals received an intraperitoneal injection of 1×10^7 pfu neu encoding recombinant vaccinia virus (rVV) either 2 weeks prior to or 2 weeks following neu-pcDNA3; 5) Animals received 100 mg CTLA-4 blocking antibody 1 day prior to and 2 days following neu-pcDNA3 to prevent T cell inhibitory signaling through CTLA-4; 6) Animals were given plasmid encoding neu fused with LAMP-1 so that neu expression was directed to the MHC class II antigen processing pathway.

b. Results.

Despite the significant protection seen in FVB/N mice after a single vaccination with neu-pcDNA3, we saw no protection from tumor challenge in transgenic mice following vaccination (**Figure 1**). We attempted to boost the immune response through multiple vaccinations or through the spatial distribution of the vaccine among several sites that drain different lymph node regions, but were unable to enhance the anti-tumor response (data not shown). Costimulation, the addition of neu rVV, CTLA-4 blockade, and the enhancement of MHC class II presentation were similarly ineffective.

c. Discussion.

The discrepancy in the results seen in FVB/N mice relative to transgenic mice is clearly the result of immunologic tolerance to HER-2/neu in the latter case. In addition, the size of the neu-pcDNA3 plasmid, 9.5 kb (5.5 kb vector + 4 kb neu cDNA), is likely to severely limit the efficiency of transfection *in vivo*. The low-level transfection after DNA vaccination in FVB/N mice is sufficient to generate neu-specific immunity, but insufficient to overcome tolerance in neu transgenic mice.

d. Future Studies.

Our laboratory is currently conducting experiments designed to identify specific peptide epitopes recognized by neu-specific T cells (We have generated a panel of CD4 and CD8 T cell clones specific for HER-2/neu). Once these peptide epitopes are known, we will generate new plasmids constructs encoding the relevant peptide(s). The smaller size of these constructs relative to that encoding the full-length protein, may result in greater transfection efficiency *in vivo* and, consequently, greater immune response to tumor in transgenic mice.

e. Recommendations in relation to the Statement of Work.

We expect to complete screening the entire HER-2/neu gene for the specific epitopes recognized by the CD4 and CD8 clones. This study is complicated by the fact that there are not previously published epitope motifs for the H-2q background mouse. We may be able to construct minigenes containing the motifs by the end of year 4.

2. Generation of Recombinant Vaccinia Vectors.

Progress Report for the funding period 9/98 to 10/99.

At the end of year two of this proposal, we had successfully generated a rVV encoding the full-length HER-2/neu protein. In addition, we had prepared a fusion of HER-2/neu with LAMP-1 and cloned this product into the pSC11 vaccinia recombination vector for the generation of a second rVV. The third year of this proposal was spent preparing the second rVV encoding the MHC class II-directed neu-LAMP-1 fusion protein.

a. Procedures/Results

To generate the neu-LAMP rVV, we first infected 3 x 10^5 CV-1 cells with 3 x 10^5 pfu wild type vaccinia virus in a six-well plate. After a two-hour incubation period, the infected CV-1 cells well were transfected with 20 μg neu-LAMP/pSC11 plasmid by the calcium phosphate precipitation method. The vaccinia-infected CV-1 cells were then grown overnight at 37°C at 5% CO_2 to allow replication of the virus. The cells and debris were then collected, centrifuged and resuspended in minimal essential media with 2.5% bovine calf serum (MEM 2.5). The cell suspension containing wild-type and recombinant vaccinia virus was subjected to three freeze-thaw cycles and then sonicated for 3 min on ice. Serial dilutions of the vaccinia virus were then used to infect TK- cells and allowed to incubate in MEM 2.5 supplemented

with 50 μg/ml 5-bromo-deoxyuridine (BrdU). Because the pSC11 vector is designed to insert within the vaccinia thymidine kinase gene, TK- cells harboring wild-type vaccinia (i.e. cells expressing virally encoded thymidine kinase) will die when placed in media containing BrdU. In this way, it is possible to isolate plaques formed exclusively by recombinant vaccina virus, in which the thymidine kinase gene has been interrupted by the insertion of neu-LAMP. Neu-LAMP rVV was subjected to three rounds of plaque purification prior to amplification. The resulting neu-LAMP rVV was titered to determine pfu/ml.

c. Discussion.

We now have recombinant vaccinia virus encoding normal rat neu as well as the neu-LAMP fusion protein. We have already tested the neu rVV in vivo (see section 3) and we are currently in the process of amplifying the neu and neu-LAMP rVV to levels sufficient for experiments designed to compare their in vivo activities in transgenic mice.

d. Future Studies.

We are currently testing the neu and neu-LAMP rVV for *in vitro* expression using fluorescent microscopy to localize the HER-2/neu gene product on the surface (neu rVV) or in the lysosomal compartment (neu-LAMP rVV) of infected cells. NIH3T3 cells will be infected with neu rVV, neu-LAMP rVV, or HA rVV at a multiplicity of infection of 3 pfu/cell. After 18-24 hrs, cells will be harvested, fixed with formaldehyde, and permeablized with saponin. After spinning the cells onto slides, the cells will be incubated with antibody against rat neu and LAMP-1, using antibody against mouse MHC class I as a positive control and antibody against human MHC class I as a negative control. A fluorescent-labeled secondary antibody will then be used to visualize neu expression in these cells. Once neu expression is confirmed *in vitro*, we will compare the ability of neu rVV and neu-LAMP rVV to generate protective anti-tumor immunity in transgenic mice alone and in combination (see section 3)

e. Recommendations in relation to the Statement of Work.

We expect to complete in vitro testing of the HER-2/neu – LAMP-1 fusion protein in the first 3 months of the fourth year. We will complete testing of the construct in vivo by the end of the fourth year as discussed below in section 3.

3. Testing of Neu-specific Vaccines *in vivo.*

We have previously demonstrated the induction of protective anti-tumor immunity in FVB/N mice following neu-specific plasmid DNA and irradiated GM-secreting whole-cell vaccinations. During the previous year of funding we have applied these vaccine techniques to the protection of transgenic mice from an NT challenge. In addition, we have titered neu rVV in FVB/N mice to determine the minimum dose required for tumor protection and evaluated the effectiveness of neu rVV in transgenic mice.

a. Procedure.

Titration of neu rVV. Our initial experiments using rVV indicated that some occurred at higher doses (>3 x 10^7 pfu). For this reason, we set out to determine the minimum effective dose of neu rVV that could be safely used. FVB/N mice (5 per group) were given neu rVV at doses ranging from 1 x 10^6 to 3 x 10^7 pfu per animal. Control animals received 1 x 10^7 pfu encoding an irrelevant influenza protein (HA rVV). Two weeks following vaccination, animals were given a subcutaneous NT challenge (5 x 10^6 cells per animal) and were monitored for the development of palpable tumors.

Tumor protection in transgenic mice using neu rVV. Transgenic mice, 5 per group, were vaccinated two weeks prior to tumor challenge. Groups included a control (3 x 10^7 pfu HA rVV), neu (2 x 10^7 pfu neu rVV, 1 x 10^7 pfu HA rVV), neu/GM (2 x 10^7 pfu neu rVV, 1 x 10^7 pfu murine GM-CSF rVV), neu/B7-1 (2 x 10^7 pfu neu rVV, 1 x 10^7 pfu murine B7-1 rVV) and neu/B7-2 (2 x 10^7 pfu neu rVV, 1 x 10^7 pfu murine B7-2 rVV). Tumor challenge consisted of 5 x 10^4 NT cells given subcutaneously in the right hind limb. Animals were monitored for the development of palpable tumors.

Because initial experiments indicated that a single vaccination was not sufficient for tumor protection, experiments were performed to determine whether multiple vaccinations were capable of inducing a protective anti-tumor response. Transgenic mice, 10 per group, were vaccinated with 3 x 10^7 pfu neu rVV at days –28, -21, -14 and –7 or days –21 and –7 prior to NT challenge (5 x 10^4 per animal). A vernier caliper was used to take two measurements, 90° apart, for each tumor. Values were then averaged to determine the mean tumor diameter for each group. Animals were sacrificed when tumor diameter exceeded 12 mm. Animals with tumor diameter less than 5 mm were scored as tumor-free.

Whole-cell vaccination. Transgenic mice, 10 per group, were given subcutaneous injections of 1 x 10^6 irradiated cells (NT, NT/GM, or 3T3-neu/GM) each in both forelimbs and the left hind limb on days –28 and –14 prior to tumor challenges (control animals received Hanks Balanced Salt Solution). On day 0, animals received a subcutaneous NT challenge (5 x 10^4 cells) in the right hind limb and were monitored for the development of palpable tumors.

To verify that the observed tumor protection in both FVB/N and transgenic mice was, in fact, T cell mediated, the above experiment was performed in combination with T cell depletion. Animals were given i.p. injections of antibody against CD4 (GK1.5), CD8 (2.43), NK cells (PK 136), or irrelevant antibody against human MHC class I (W 632) twice weekly (100 mg/injection). The depletion of $CD4^+$ and $CD8^+$ T cell subsets was verified by FACS analysis of splenic T cells prior to vaccination. Depletion was maintained throughout the experiment.

b/c. Results and Discussion.

Titration of neu rVV. Using non-toleragenic FVB/N mice, we showed that protection from tumor challenge was possible at neu rVV doses as low as 1 x 10^6 (**Figure 2**). Tumor protection was greatest at the highest dose of neu

rVV used (3×10^7 pfu) with no mortality occurring in any group. From these experiments, we established 3×10^7 pfu rVV as the maximum allowable dose.

Tumor protection in transgenic mice using neu rVV. The first series of experiments in transgenic mice were performed to determine whether costimulation through the administration of neu rVV in conjunction with rVV expressing GM-CSF, B7-1 or B7-2 could enhance in vivo T cell priming, leading to better protection from tumor challenge. The data, shown in **Figure 3**, indicated that after a single vaccination with neu rVV, tumor growth was delayed relative to control animals, but the level of protection was not significant. Furthermore, co-administration of the immunostimulatory molecules GM-CSF, B7-1 or B7-2 did not enhance anti-tumor immunity.

These findings led us to evaluate the effectiveness of multiple neu rVV vaccinations in transgenic mice. In **Figure 4**, we show that tumor growth is slowed after 2 vaccinations, relative to control animals, and slowed further after 4 vaccinations. Depicted in a Kaplan-Meyer Survival curve (**Figure 5**), animals show significant protection from NT challenge after four weekly vaccinations with neu rVV relative to control animals ($p<0.01$). As was the case with plasmid DNA vaccination, high levels of neu expression appears to be required for protection in transgenic mice. Multiple vaccinations with high doses of rVV expressing neu, combined with the adjuvant properties associated with the anti-vaccinia virus response in vivo, was sufficient to overcome tolerance to HER-2/neu in transgenic mice. However, further manipulation of the immune response may be necessary for sustained anti-tumor immunity.

Whole-cell vaccination. In order to generate GM-CSF-secreting cell lines for neu-specific vaccination, NT cells and 3T3-neu cells were retrovirally transduced with the cDNA encoding mouse GM-CSF NT/GM and 3T3-neu/GM). After vaccination with a neu+ GM-secreting cell line, transgenic mice were protected from NT tumor challenge (**Figure 6**). Best results were obtained using the NT/GM cell line ($p<0.0001$), but significant protection was also seen after NT ($p<0.003$, data not shown) and 3T3-neu/GM ($p<0.005$). The fact that significant protection was seen after vaccination with neu rVV or 3T3-neu/GM indicates that HER-2/neu, specifically, is the *in vivo* tumor rejection antigen. Furthermore these data are consistent with the idea that neu-specific T cells in transgenic mice have escaped central tolerance and can be activated *in vivo*.

We next performed experiments designed to verify that the observed anti-tumor immunity was T cell-mediated. After the depletion of $CD4^+$ T cell, $CD8^+$ T cell or NK cell subsets, both FVB/N and transgenic mice received a 3T3-neu/GM vaccine, followed by NT challenge 14 days later. The data, summarized in **Table I**, show that mice depleted of $CD4^+$ T cells develop tumors with kinetics that are similar to that seen in unvaccinated, undepleted controls. The deletion of $CD8^+$ T cells has a less dramatic effect on tumor growth, though NT growth is statistically distinct from vaccinated, undepleted animals, and is very similar to unvaccinated, undepleted controls. The tumor-free survival of animals given the NK cell-depleting

antibody PK 136 and vaccine were identical to that of undepleted, vaccinated mice. These data establish the importance of both $CD4^+$ and $CD8^+$ T cells in inducing neu-specific immunity and clearly indicate that the protection from tumor challenge is T cell-mediated.

d. Future Studies.

Our preliminary data clearly indicate that neu-specific T cell function is impaired in HER-2/neu transgenic mice relative to non-toleragenic FVB/N animals. Although both $CD4^+$ and $CD8^+$ T cells are critical for inducing potent anti-tumor immune responses, this proposal will concentrate on the $CD8^+$ CTL effector cells. We hypothesize that in the HER-2/neu transgenic mice the high avidity neu-specific T cells, which account for the protective effects seen in FVB/N mice, have undergone central deletion in the thymus during T cell development. Lower avidity neu-specific T cells, however, escape central deletion and remain active. These T cells likely have more stringent requirements for activation and effector function and, in addition, may undergo mechanisms of peripheral tolerance to HER-2/neu, further dampening their response. This model is consistent with published reports carried out in animal models of tolerance, where lower avidity T cells were shown to persist in a toleragenic host (34-37). Importantly, these T cells can still be activated sufficiently to result in protection of mice from the development of an antigen-expressing tumor cell line (34). Using an ovalbumin model of autoimmune diabetes, Kurts and coworkers demonstrated that $CD8^+$ Ova-specific T cells, when transferred into mice expressing ovalbumin specifically in the β-islets of the pancreas, were tolerized in an APC-dependent fashion (38). However, when ova-specific $CD4^+$ T cells were co-transferred, the ova-specific CTL were not deleted and diabetes resulted (38), thus implicating a requirement for T cell help in the induction of the autoimmune response. It is likely that the tolerization of neu-specific $CD4^+$ T cells in the transgenic mice results in a lack of T cell help *in vivo*, thereby diminishing both the humoral and cellular responses to tumor. In order to enhance to the anti-tumor response seen in transgenic mice, we will modify our existing vaccine strategies to target four different mechanisms of T cell activation.

First, we will seek to enhance $CD4^+$ T cell helper function by re-directing antigen expression to the MHC Class II compartment. Typically, MHC class II antigens are derived from proteins that are endocytosed by an APC and degraded in the lysosome, where the resulting peptides are loaded onto MHC class II molecules for presentation to $CD4^+$ T cells. Using the neu-LAMP rVV, we expect to boost the $CD4^+$ T cell response *in vivo* and thus, enhance the overall immune response to tumor. Transgenic mice will be given a series of four weekly vaccinations with 3×10^7 pfu neu-L rVV followed by an NT challenge, given s.c., 7 days after the final vaccination. Animals will then be monitored for the formation of palpable tumors Control animals will receive HA rVV.

We will also focus on providing better co-stimulation *in vivo* to facilitate a more vigorous immune response *in vivo*. The initiation of a CTL

response *in vivo* occurs in multiple steps. The first step is the internalization of antigen by immature APC. When this occurs in the presence of pro-inflammatory cytokines such as γ-IFN or TNF-α, the APC mature and migrate to draining lymph nodes or to the spleen where they interact with T cells. CD40, a member of the tumor necrosis factor receptor family, is found on various APC such as DC, B cells, and macrophages, while the ligand for CD40, CD154, is expressed predominantly on $CD4^+$ T cells (39,40). T cell help, in the form of CD40 ligation, results in the maturation of APC and facilitates the activation of $CD8^+$ T cells. This is accomplished through the enhancement of antigen processing and presentation, the up-regulation of B7 molecules, as well as the adhesion molecule ICAM-1, and the production of inflammatory cytokines such as TNF-α and IL-12 by the APC (41, 42). Commercially available agonist antibodies against CD40 have been used successfully *in vivo* to overcome immune tolerance (43) and to overcome the absence of $CD4^+$ T cell help in the generation of anti-tumor immunity when given in combination with an antigen-specific vaccine (44-46). By augmenting our neu rVV vaccine strategy with anti-CD40 agonist antibody, we expect to induce a more potent *in vivo* tumor eradication effect through better priming of the immune response as well as the prevention of the reacquisition of tolerance after activation.

Transgenic animals will receive four weekly vaccinations with neu rVV as described above. In addition, animals will be given i.v. injections of the anti-CD40 agonist antibody FGK-45 (100 μg/day) on day 0 and day 2 following each vaccination. An NT tumor challenge will be given 7 days following the final vaccination and animals will be monitored for the formation of palpable tumors.

During T cell-APC interaction, the ligation of CD28 on the surface of the T cell by B7 molecules on the APC is absolutely required for activation. Rapidly following CD28 ligation, however, T cells up-regulate the surface expression of CTLA-4, a structurally similar receptor molecule that also binds for B7. However, the engagement of CTLA-4 by B7-1, unlike CD28 engagement, leads to diminished IL-2 expression and decreased proliferation (47-49). CTLA-4 ligation appears to serve separate functions on resting and activated T cells. Resting T cells, which are small in size and lack activation markers such as CD25 and CD69, express very low levels of CTLA-4 on their surface. These cells, resting memory cells in particular, are very sensitive to CTLA-4 crosslinking (50, 51). Thus, in resting T cells CTLA-4 appears to provide a threshold for activation, preventing activation in the presence of low strength TCR signals. Upon activation, T cells upregulate CTLA-4, which has a higher affinity for B7 then CD28. By out-competing CD28 for B7 occupancy, CTLA-4 decreases signaling through CD28. Signaling through CTLA-4 then leads to apoptosis of the activated T cells due to the lack of IL-2. Thus, in activated T cells, CTLA-4 may serve to terminate effector function. By blocking signaling through CTLA-4 *in vivo*, a number of groups have demonstrated that it is possible to induce regression and protection from

subsequent challenge with a highly immunogenic tumor using anti-CTLA-4 blocking antibodies (52, 53). Hurwitz and colleagues were able to generate protective immunity from a weakly immunogenic tumor when anti-CTLA-4 was given with an irradiated tumor cell vaccine, where CTLA-4 alone and vaccine alone were ineffective (54). We propose to administer commercially-available anti-CTLA-4 blocking antibody alongside neu rVV in order to enhance the modest protective effects observed previously by lowering the threshold for activation and by limiting the termination of the immune response by signaling through CTLA-4.

Transgenic animals will be vaccinated as described above. In addition to neu-specific vaccination, animals will be given i.p. CTLA-4 blocking antibody (100 μg/day) on day 2 and day 4 following vaccine as well as day 2 and day 7 following NT challenge. By blocking signaling through CTLA-4 after vaccination, we expect to enhance neu-specific T cell proliferation and differentiation. The re-administration after tumor challenge may be necessary to prevent activation-induced cell death, which may occur following encounter with tumor-derived HER-2/neu. Isotype-matched irrelevant antibody will be given to the control group and animals will be monitored for the formation of palpable tumor.

OX-40 is a receptor in the tumor necrosis factor receptor family that is found on activated $CD4^+$ T cells. OX-40 induction occurs by 24 hr after stimulation, peaking at between 24 and 72 hr. Upon re-stimulation, OX-40 appears on the surface by 4 hr (55, 56). The ligation of OX-40 on $CD4^+$ T cells by OX-40 ligand (OX-40L) on APC in addition to TCR engagement and CD28/B7-1 interaction has been shown to greatly enhance IL-2 production and proliferation of T cells in culture (57-59). In an animal model of experimental autoimmune encephalitis (EAE), auto-reactive T cells isolated from the spinal cord of immunized rats showed increased surface expression of OX-40 (60). The *ex vivo* stimulation of these auto-reactive T cells with anti-CD3 and anti-OX-40 agonist antibodies induce EAE when transferred into naïve hosts (60), while the *in vivo* administration of toxin-conjugated anti-OX-40 antibody successfully ameliorated EAE in a separate study (61). Because priming through OX-40 has been demonstrated to promote clonal expansion, enhanced effector function, and prolonged survival of auto-reactive T cells in autoimmune disease, it is possible that OX-40 activation may significantly enhance our vaccine strategies in HER-2/neu transgenic mice. The *ex vivo* stimulation of tumor-specific $CD4^+$ T cells with commercially available anti-OX-40 agonist prior to transfer into tumor bearing host has been used to successfully treat sarcoma in mice (Andrew Weinberg, personal communication). In addition, the *in vivo* administration of OX-40 agonist antibody was demonstrated to be effective in murine models of melanoma, breast, and renal cancer as well (Andrew Weinberg, personal communication). We hypothesize that the administration of anti-OX-40 agonist in combination with neu rVV will generate more efficient priming of neu-specific $CD4^+$ T cells *in vivo* and will lead to a more vigorous and longer-lasting tumor-specific immune response.

In addition to neu-specific vaccination, animals will receive i.p. injections of anti-OX-40 agonist antibody (150 μg/day) on day 2 and day 4 following each vaccination. As with anti-CTLA-4, it may be necessary to administer anti-OX-40 after tumor challenge in order to maintain neu-reactivity in the toleragenic HER-2/neu mice. Isotype-matched irrelevant antibody will be given to the control group and animals will be monitored for the formation of palpable tumor.

Each of the modifications described above are considered separately for the sake of simplicity. However, we expect that it may be necessary to combine any or all of these approaches in order to achieve full tumor eradication *in vivo*. In addition, we expect that data supporting new, more effective methods for vaccine enhancement may arise during the funding period of this grant proposal, which would then be considered as well.

Any or all treatment methods described above that show increased benefit in the tumor challenge experiments will be adapted to the prevention of spontaneous tumor development in the HER-2/neu transgenic mice. We have been able to delay the onset of spontaneous tumor development using vaccine strategies that showed promise in early tumor challenge experiments (see below). However, we have been unable to sustain the immune response to tumor. The treatment methods described above are designed to generate enhanced tumor-specific effector function and to sustain the anti-tumor response in the HER-2/neu transgenic mice, despite the mechanisms of peripheral tolerance that are ongoing in the transgenic mice.

e. Recommendations in relation to the Statement of Work.

We expect to complete the proposed studies described above in year four. By the end of year four, we hope to have identified at least one immune modulating agent that enhances the potency of our most potent vaccinia vaccine.

4. Evaluate recombinant vaccines for synergy with ablation of the mammary ductal system in preventing breast cancer.

We have developed a method for the direct cannulation of the mammary duct for the delivery of retrovirus, rVV or recombinant adenovirus (rAd) designed to alter the local environment of the breast epithelium to create a more immnostimulatory environment. In the previous year of funding, this technique was used in combination with neu rVV vaccination for the prevention of spontaneous tumor development in transgenic mice.

a. Procedures.

Four groups consisting of 12 post-lactational transgenic mice (~18 weeks of age), each, were used in this experiment. The Vac Only and Vac + Abl groups each received weekly i.p. injections of 3×10^7 pfu neu rVV beginning at 19 weeks and continuing until 23 weeks. Animals receiving ablation of the mammary tissue (Vac + Abl and Abl Only) were given 1×10^6 pfu neu rVV and 1×10^5 pfu B7-1 rVV through cannulation of the mammary

duct (10 ml per teat, $1x10^7$ pfu neu rVV and $1x10^6$ pfu B7-1 rVV total per animal) on week 21. Animals were then monitored for the development of spontaneous mammary tumors.

b/c. Results and Discussion.

In all treatment groups, a significant delay in the onset of tumor development was seen (**Figure 7**, $p = 0.02$ at 80 days post-vaccine). We chose to add neu rVV and B7-1 rVV to the mammary duct to try to generate a local neu-specific inflammatory response there. Because the overall dose of neu rVV involved with the mammary ablation (1×10^7 pfu) approaches that of a single i.p vaccination (3×10^7 pfu), it is likely that the ablation alone acted as a vaccine. Thus, it is impossible to determine whether the delay in tumor onset observed in the Abl Only group is due to the ablation of mammary tissue, or to the effects of the neu-specific vaccination. In future experiments, we will not use a neu-expressing vector in the mammary duct cannulation to facilitate data interpretation.

d. Future studies.

Because the natural occurrence of spontaneous tumors in the transgenic mice occurs over an extended period of time, larger groups (>16 animals) are required in order to determine statistical significance. Thus, we will use larger groups in future studies. In addition to increasing group size, we will incorporate the co-stimulatory vaccine modifications described above in order to generate longer-lasting *in vivo* responses. Because neu-specific T cell activated in response to vaccination may be re-tolerized in transgenic mice, it may be necessary to continue bi-weekly vaccinations throughout the experiment to further sustain the anti-tumor response. Finally, In order to create a more immunostimulatory environment within the mammary epithelium, we will add recombinant adenovirus encoding GM-CSF and/or IL-2 directly into the mammary duct. We will determine whether a single cannulation is sufficient for enhanced anti-tumor immunity, or whether repeated injections of cytokine-encoding rAd are necessary.

e. Recommendations in relation to the Statement of Work.

We expect to identify at least one combination of vaccine plus immune modulating agent that can significantly delay or prevent entirely the development of naturally developing tumors.

7. KEY RESEARCH ACCOMPLISHMENTS.

- Demonstration that HER-2/neu is a tumor rejection antigen in the HER-2/neu transgenic mouse model of breast cancer
- The identification of a vaccine approach that significantly delays the development of spontaneously arising tumors in antigen-tolerized mice
- Development of several recombinant HER-2/neu specific vaccines
- Development of five HER-2/neu expressing transplantable cell lines
- Development of a panel of CD4 and CD8 HER-2/neu specific T cell clones

8. REPORTABLE OUTCOMES

1. A manuscript has been submitted which is the first description of the immunologic characterization of the HER-2/neu transgenic mouse model developed by William Muller. This manuscript demonstrates that HER-2/neu can serve as an in vivo tumor rejection target in these mice. In addition, spontaneous tumors can be significantly delayed by vaccination (See manuscript in appendix).
2. Dr. Todd Reilly, the postdoctoral fellow working on this proposal was the recipient of the AACR fellowship for Prevention in 1998. Dr. Reilly presented the data described in this progress report at the 1999 AACR meetings in April in Philadelphia, PA. He was invited to present his abstract at a minisymposium on immunotherapy.
3. Five transplantable HER-2/neu expressing mammary cell lines were developed from spontaneously arising mammary tumors in these mice.
4. A phase I trial based on data from this grant is being designed.
5. Dr. Reilly has applied for an NIH sponsored faculty career development award based on work performed on this grant.

9. CONCLUSIONS.

This proposal seeks to develop a vaccine strategy that can specifically generate an immune response to ductal cells (the normal cells from which the majority of breast cancers arise), to preneoplasias, and to incipient tumor cells, by targeting common antigens expressed by these cells, as an alternative therapy for preventing breast cancer development. We are employing the HER-2/neu transgenic mouse model of breast cancer to evaluate vaccine strategies developed in our laboratory. We have demonstrated that it is possible to generate a neu-specific immune response in transgenic mice, despite tolerance to HER-2/neu. Furthermore, neu-specific vaccination is sufficient to delay the onset of spontaneous tumor development. In the next year, we will test various methods designed to enhance the magnitude and duration of the anti-tumor response. These vaccine strategies will then be applied to the prevention of spontaneous tumor development in conjunction with methods designed to create a more immunostimulatory local environment within the mammary epithelium.

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9. APPENDICES.

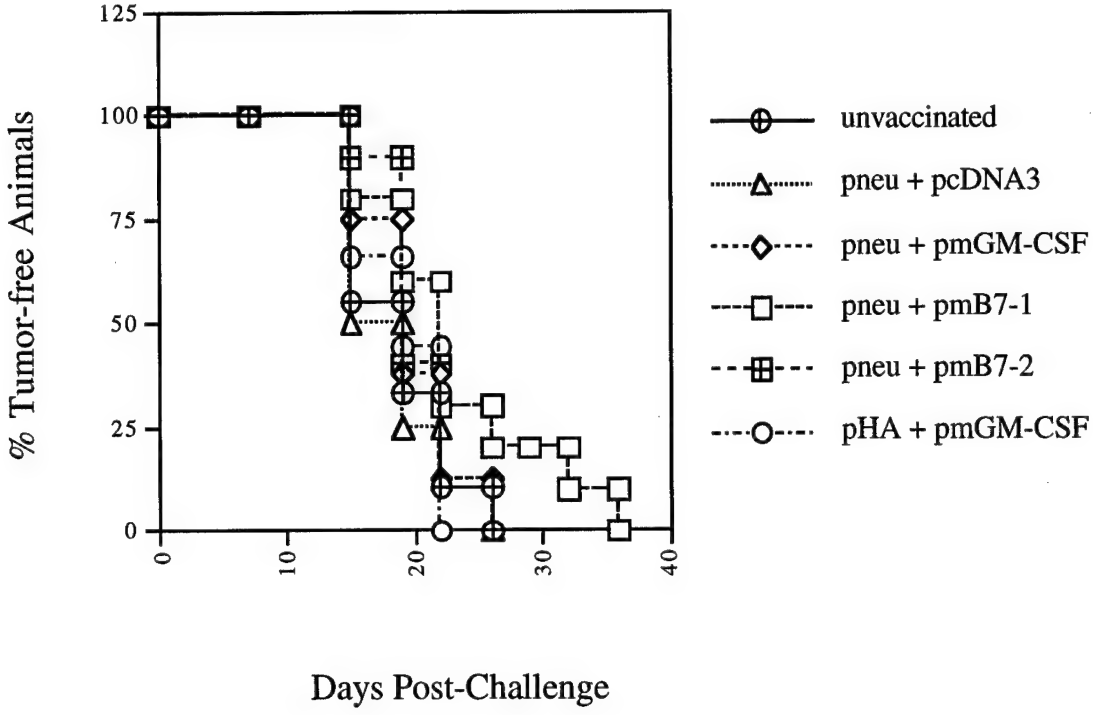

Figure 1. Transgenic mice were given an intradermal vaccination consisting of a total of 1 µg plasmid DNA (groups indicated on graph). Animals received a subcutaneous NT challenge in the right hind limb 14 days post-vaccination and were monitored for the formation of palpable tumors.

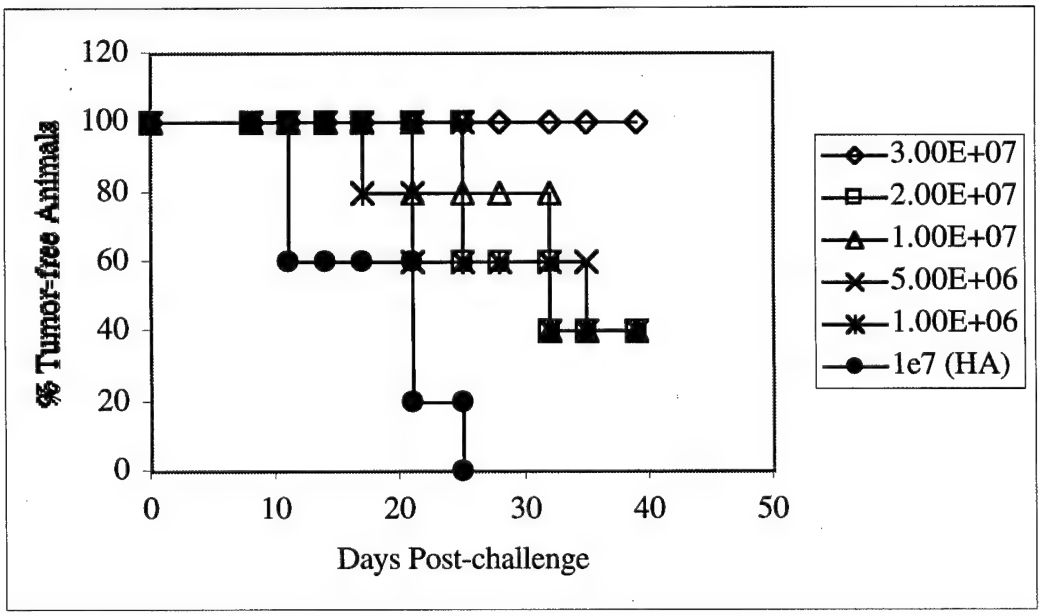

Figure 2. FVB/N mice were given an i.p. injection of the indicated dose of neu rVV (control animals received 1×10^7 pfu HA rVV) followed by NT challenge 14 days later. Animals were then monitored for the development of palpable tumors.

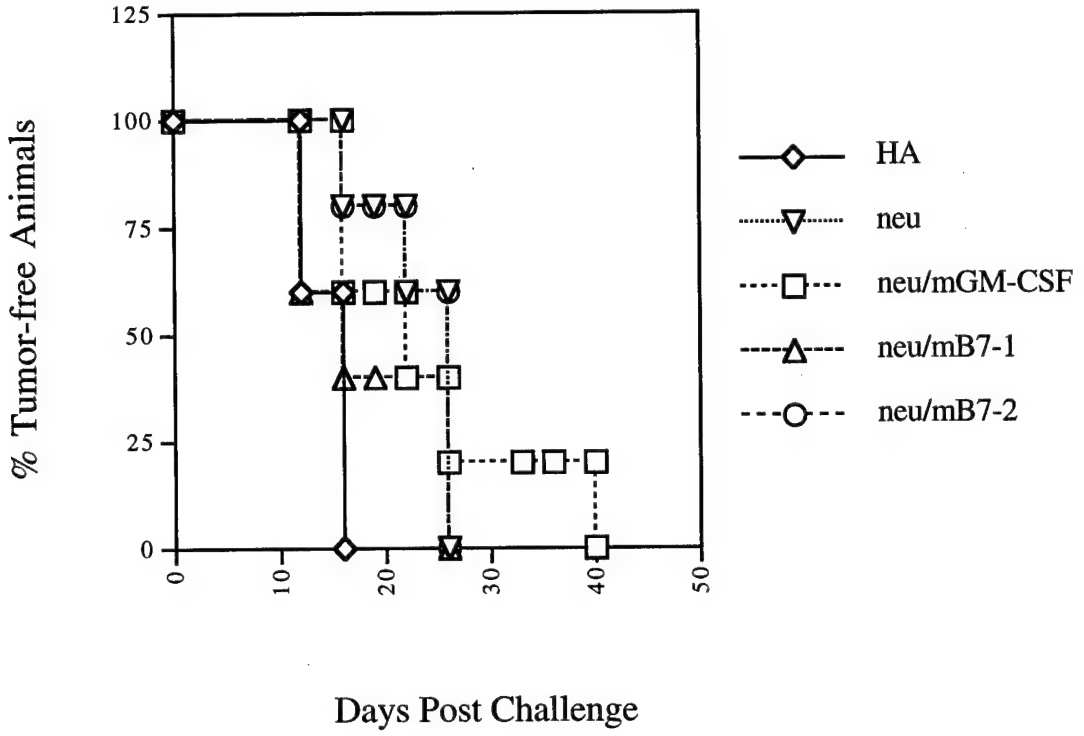

Figure 3. Transgenic mice were vaccinated with the indicated rVV or combination of rVV (total dose 3 x 10^7 per animal) followed 14 days later by NT challenge. Animals were monitored for the development of palpable tumors.

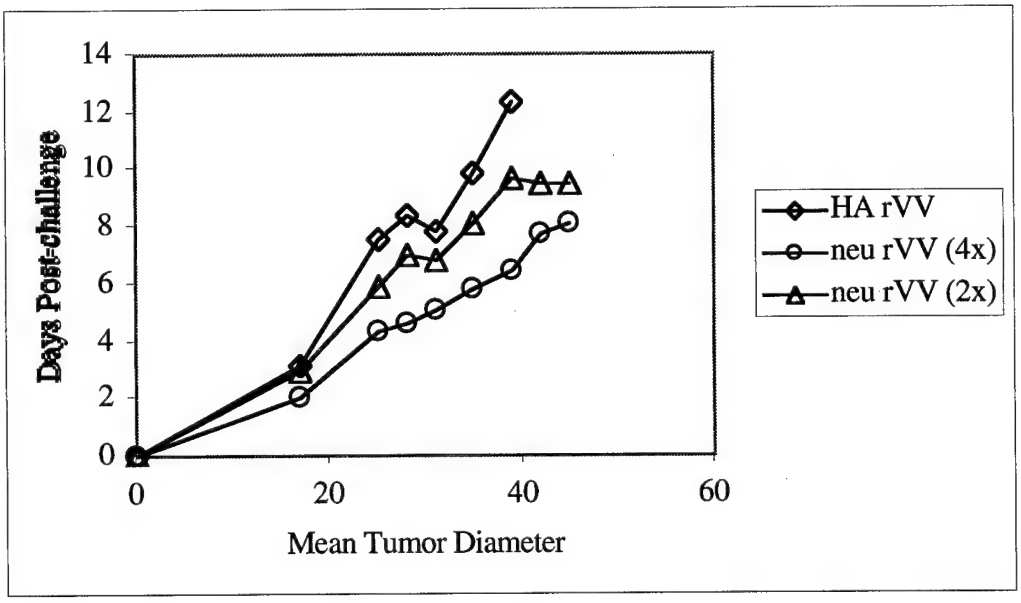

Figure 4. Transgenic mice were given vaccinations consisting of 3 x 107 pfu neu rVV on days –28, -21, -14, and –7 (4x) or days –14 and –7 (2x) prior to NT challenge. Mean tumor diameter was determined in two dimensions for each animal, and the average determined for each group over time. Control animals received HA rVV on the same schedule as the 4x group.

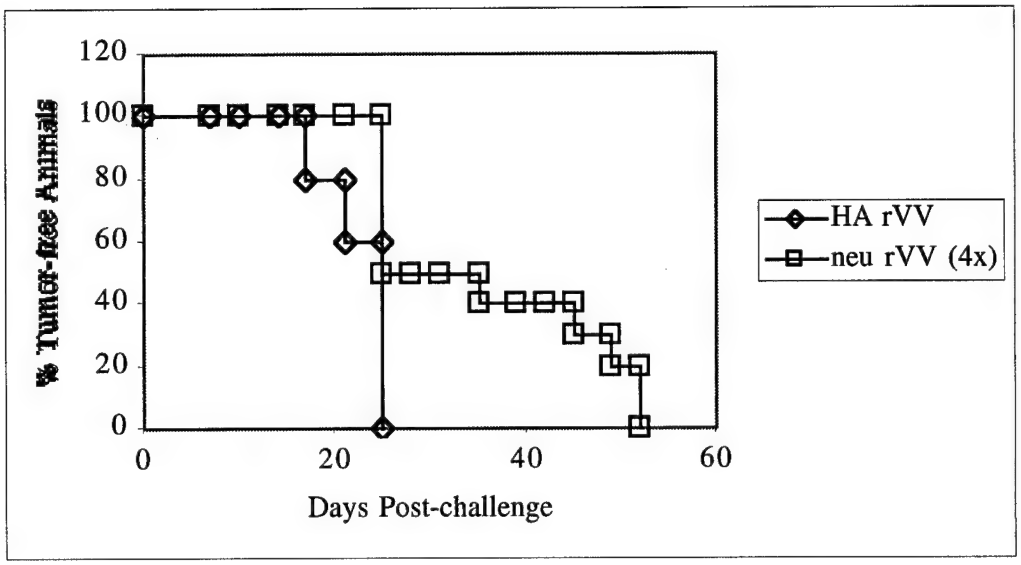

Figure 5. Transgenic mice were given vaccinations consisting of 3 x 107 pfu neu rVV on days –28, -21, -14, and –7 (4x) prior to NT challenge. Control animals received HA rVV on the same schedule as the 4x group. Vaccinated animals were protected from NT challenge relative to controls ($p<0.01$).

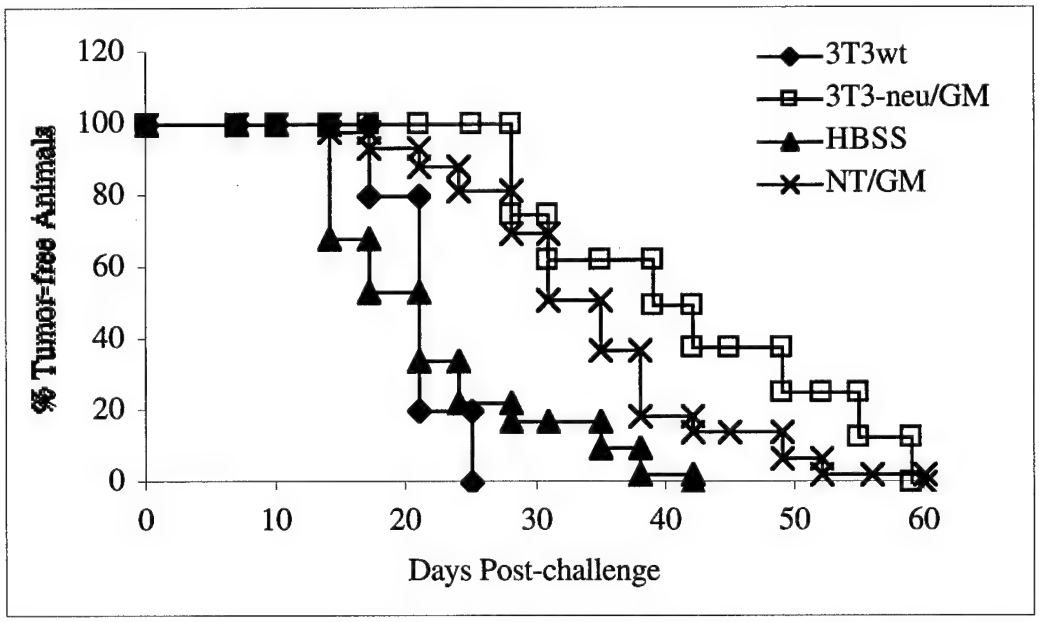

Figure 6. In separate experiments, transgenic mice were vaccinated with 3T3wt, 3T3-neu/GM, Hanks Balanced Salt Solution (HBSS), or NT/GM followed by NT challenge 14 days later. 3T3-neu/GM (n = 30) and NT/GM (n = 55) vaccinated animals showed significant protection from tumor challenge ($p<0.005$ and 0.0001, respectively) relative to control animals (3T3wt and HBSS respectively).

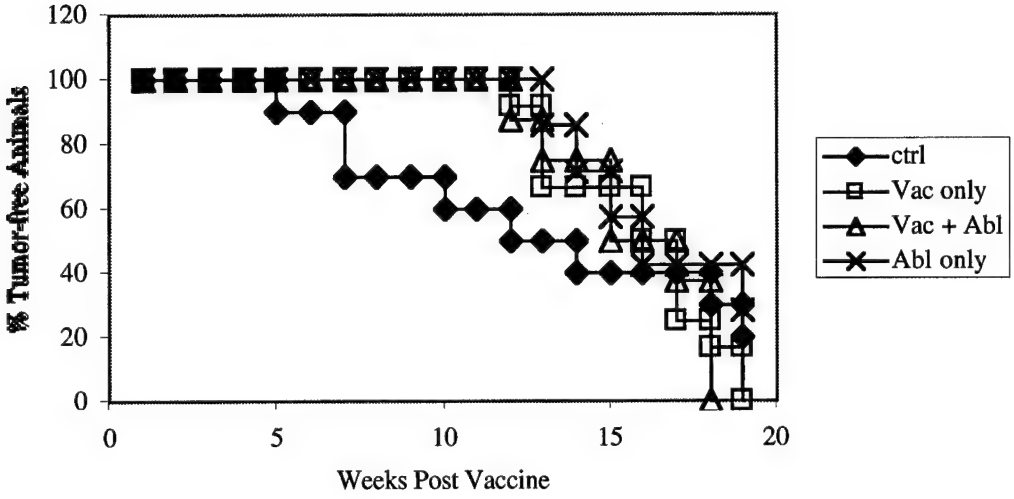

Figure 7. Groups consisting of 12 post-lactational transgenic females received 5 weekly vaccinations with 3 x 107 pfu neu rVV beginning at approximately 18 weeks of age (Vac only and Vac + Abl). In addition, local ablation was performed using neu rVV and B7-1 rVV at 22 weeks of age (Vac + Abl and Abl only). Control animals were unmanipulated. The percentage of tumor free animals is expressed as a function of time after the initial vaccination. At approximately week 12, vaccinated animals showed significant protection from spontaneous tumor formation relative to controls ($p = 0.02$).

Table I. Effects of T cell Depletion on Anti-tumor Immunity

Mice	Group	% Tumor-free (day 28)	P value (vs. control)
FVB/N	no dep, no vac	0	---
FVB/N	CD4 dep, vac	15	0.05
FVB/N	CD8 dep, vac	60	0.007
FVB/N	NK dep, vac	100	>0.0001
FVB/N	no dep, vac	100	>0.0001
HER-2/neu	no dep, no vac	0	---
HER-2/neu	CD4 dep, vac	0	0.31
HER-2/neu	CD8 dep, vac	40	0.02
HER-2/neu	NK dep, vac	68	0.004
HER-2/neu	no dep, vac	75	0.007

Animals were depleted of the indicated cell populations (verified by FACS analysis) prior to vaccination with 3T3-neu/GM followed 14 days later by NT challenge. Tumor-free survival at day 28 post-challenge and significance (Logrank Test) relative to no dep, no vac is indicated.

HER-2/neu is a Tumor Rejection Target in the HER-2/neu Transgenic Mouse Model of Breast Cancer[1]

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Running Title: HER-2/neu is target antigen in neu transgenic mice

Key words: tumor immunology; tolerance; transgenic mice; HER-2/neu; tumor antigen.

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[3] The abbreviations used are: neu-N, normal HER-2/neu; neu-T, mutated transforming HER-2/neu; MHC, major histocompatability complex; TCR, T cell receptor; CTL, cytolytic T

lymphocyte; IgG, immunoglobulin G; IFN-γ, interferon gamma; IL, interleukin; NK cell, natural killer cell; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; ELISPOT, enzyme-linked immuno-spot assay; rVV, recombinant vaccinia virus; pfu, plaque-forming unit; mGM-CSF, murine granulocyte-macrophage colony-stimulating factor; HBSS, Hank's balanced salt solution; TA; tumor antigen; HA, hemagglutinin; NT, neu tumor; NT/GM, neu tumor producing mGM-CSF; 3T3, NIH-3T3; 3T3/GM, 3T3 cells producing mGM-CSF; 3T3-neu, ATCC line CRL-1915; 3T3-neu/GM, 3T3-neu cells producing mGM-CSF.

[4]Reilly, R. T. and Jaffee, E. M. Active Immunization Against the HER-2/neu Proto-oncogene Demonstrates the Importance of Both Antibody- and T cell-mediated Responses in Tumor Eradication., in preparation.

ABSTRACT

HER-2/neu transgenic mice, which express the non-transforming rat proto-oncogene, develop spontaneous focal mammary adenocarcinomas beginning at approximately 5 to 6 months of age. The development and histology of these tumors bears a striking resemblance to what is seen in patients with breast cancer. In addition, HER-2/neu transgenic mice, like human breast cancer patients, exhibit tolerance to the transgene, making this an ideal model in which to test immunotherapeutic approaches to treat and even prevent breast cancers. We have characterized the immunologic responses to HER-2/neu in this animal model. HER-2/neu-positive tumor lines, which were derived from spontaneous tumors that formed in HER-2/neu transgenic animals, are highly immunogenic in parental, FVB/N mice. In contrast, a 100-fold lower tumor challenge is sufficient for growth in 100% of transgenic animals. Despite significant tolerance to the transgene, HER-2/neu-specific immune responses can be demonstrated in HER-2/neu transgenic mice prior to vaccination. Both cellular and humoral HER-2/neu-specific responses in transgenic mice can also be boosted with HER-2/neu specific vaccination, although to a significantly lesser degree than what is observed in FVB/N mice, indicating that the T cells involved are less responsive than in the non-tolerigenic parental strain. Using irradiated whole-cell and recombinant vaccinia virus vaccinations, we are able to protect HER-2/neu transgenic mice from a subcutaneous HER-2/neu-expressing tumor challenge. T cell depletion experiments demonstrated that the observed protection is T cell dependent. The depletion of $CD4^+$ T cells completely abrogates the anti-tumor effect and tumors develop at the same rate as unvaccinated control mice. In contrast, $CD8^+$ T cell depletion results in abrogation of the antitumor effect following a delay in tumor development compared with control mice. These data suggest that,

despite tolerance to HER-2/neu in this transgenic model, it is possible to immunize neu-specific T cells to achieve neu-specific tumor rejection *in vivo*. Significantly, the vaccine-dependent HER-2/neu-specific immune response is also sufficient to prevent spontaneous tumor formation in these mice. This represents the first example of immune-mediated spontaneous tumor prevention in this transgenic model of breast cancer. It should be feasible to employ this model to identify more potent vaccine approaches that can treat and prevent the development of spontaneously arising mammary tumors.

INTRODUCTION

The development of vaccine approaches that induce antigen-specific anti-tumor immune responses against solid tumors is an active area of immunologic research. There are many reports demonstrating the induction of antigen-specific T cell responses potent enough to eradicate murine tumor cells that have been genetically modified to express model antigens such as β-galactosidase (1, 2), influenza A nuclear protein (3, 4), and the simian virus 40 large T antigen (5-7). While these model systems support a potential clinical role for vaccines, they fail to address issues that are critical for the translation of these approaches to the treatment of human cancer. For example, these model systems target an immune response against a protein that is not normally expressed in these animals. Therefore, tolerance to the tumor antigen is not expected to occur and create the same complications that are encountered in the treatment of human cancers. In addition, these models employ tumor lines that have been passaged indefinitely in culture and thus, may not grow *in vivo* in the same way as a tumor that arises naturally. In particular, the growth kinetics and pressures of the microenvironment may differ greatly between naturally developing tumors and transplantable cell lines. Also, these cell lines were engineered to constitutively express the target antigen. Therefore, the level of antigen expression in these lines is likely to be significantly higher than what might be encountered in a natural tumor.

More recently, many groups have reported the identification of naturally occurring murine and human tumor antigens (8-12). These antigens have been demonstrated to be T cell targets and, therefore, have the potential to serve as tumor rejection antigens *in vivo.*

Interestingly, these data unexpectedly revealed that many of these antigens, including MART-1, gp100, tyrosinase and HER-2/neu (13-19), are tumor associated or differentiation antigens rather than antigens that are only expressed by the tumor. Thus, these antigens are self-antigens against which natural mechanisms of T cell deletion or peripheral tolerance are expected to occur. These findings provide evidence that T cells that target antigens expressed by spontaneously arising tumors may be susceptible to tolerizing mechanisms in the host.

Newer *in vivo* models are being developed to specifically dissect the mechanisms of tumor antigen directed-tolerance, and to identify more potent vaccine strategies that have the potential to overcome these mechanisms of tolerance. These models fall into two categories: TA^3 transgenic mice and TCR transgenic mice. TA transgenic mice, such as those that express carcinoembryonic antigen (20-22), prostate-specific antigen (23), HA (24), Friend murine leukemis virus envelope protein (25) and MUC-1 (26) appear to be more clinically relevant models because antigen-specific tolerance has been shown to occur against the protein encoded by the transgene. However, these mice fail to demonstrate antigen-associated spontaneous tumor development. In contrast TA transgenic mice that express the v-Ha-*ras* oncogene develop spontaneous breast cancers. However, these spontaneous breast cancers are highly immunogenic and the T cell response is not directed against the transgene-encoded *ras* oncogene (27). Spontaneous tumor development is also seen in the TRAMP mouse model for prostate cancer (28, 29), however the immunologic response to tumor in this model has not been fully characterized to date. TCR transgenic mice demonstrate skewed T cell repertoires and thus the majority of T cells in these mice express a single TCR usage. T cells from these mice have successfully been used to track the fate of tumor-specific T cells in tumor bearing hosts (30-32).

As with previous models, however, these transgenic models utilize engineered tumor lines, which bear little clinical relevance to spontaneous tumors.

HER-2/neu transgenic mice developed by Muller (33) overexpress the non-transforming rat HER-2/neu cDNA (neu-N) under the control of a mammary specific promoter. As a consequence, these mice develop spontaneous focal mammary adenocarcinomas in a stochastic manner beginning at approximately 125 days, with the majority of mice developing spontaneous tumors by 300 days. Immunologic responses to neu have not been previously characterized in these mice. HER-2/neu is an attractive target for enhancing anti-tumor immunity since HER-2/neu-specific antibody (34, 35) and T cell (36-40) responses have been demonstrated in patients with HER-2/neu expressing mammary and ovarian cancers. Yet, HER-2/neu expressing tumors in these same patients continue to grow and metastasize, indicating that immune tolerance exists to the proto-oncogene. Since HER-2/neu is expressed as a transgene in neu-N mice, these animals would, likewise, be expected to demonstrate similar tolerance to HER-2/neu. Because the HER-2/neu transgenic mouse model for breast cancer so closely mimics human disease, these transgenic mice represent an ideal model in which to study various approaches for the immunotherapy of HER-2/neu over-expressing tumors in a setting that is clinically relevant. Furthermore, because HER-2/neu over-expression is an early event in oncogenic transformation in these animals (41-43), as in human disease, this model allows the evaluation of vaccine strategies for the *prevention* of spontaneous tumor formation, as well as for the treatment of existing disease.

We describe here the immunologic characterization of the HER-2/neu transgenic mouse model of breast cancer, and present data which demonstrate that it is possible to generate

protective immunity specifically directed against HER-2/neu both for the eradication of HER-2/neu-expressing tumors as well as for the prevention of spontaneous tumor development.

MATERIALS AND METHODS

Mice. HER-2/neu transgenic mice (line #202) (33) were bred to homozygosity (*neu/neu* mice) as verified by Southern blot analysis. FVB/N mice were obtained commercially from Jackson Laboratories. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Cell Lines and Media. NT cell lines were derived from spontaneous mammary tumors in female neu/neu mice. In vitro cell lines were established by digestion of spontaneous tumors with dispase (Boehringer) and collagenase (Boehringer) followed by differential trypsinization to remove fibroblasts. NT lines were grown in our defined Breast Media, which consisted of RPMI (Life Technologies) with 20% FBS (Hyclone), 1% L-Glutamine, 1% NEAA, 1% Na Pyruvate, 0.5% Pen/Strep, 0.02% Gentamycin (JRH Biosciences), and 0.2 % Insulin (Lilly), and maintained at 37°C in 5% CO_2. 3T3 cells (ATCC, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (Life Technologies) with 10% BCS, 1% L-Glutamine, 1% Na Pyruvate, 1% NEAA, and 0.5% Penicillin/Streptomycin at 37°C and 10% CO_2. The 3T3-neu cell line, ATCC CRL-1915 (ATCC, Rockville, MD), which expresses rat HER-2/neu, was grown in 3T3 media + 0.3 μM methotrexate at 37°C in 10% CO_2. NT/GM, 3T3/GM and 3T3-neu/GM cells were produced via retroviral transduction with a mGM-CSF-encoding retrovirus, as previously described (44). mGM-CSF production was assessed with a commercially available

ELISA kit (Endogen), and was determined to be 100-150 ng/10^6 cells/24 hrs for NT/GM cells and 200-250 ng/10^6 cells/24 hrs for 3T3/GM and 3T3-neu/GM cells.

Flow Cytometry Analysis. NT cell lines and explanted tumor tissue were assayed by flow using antibodies against murine MHC Class I (28-14-8), human MHC I (W632) and the Her-2/neu protein (neu Ab4, Oncogene Science). A fluorescein-conjugated goat anti-mouse IgG2a secondary antibody was used to detect expression of surface molecules. To determine neu-specific serum IgG titers, 3T3 and 3T3-neu cells were stained using mouse serum as the primary antibody. A fluorescein-conjugated goat anti-mouse IgG secondary antibody was used to detect bound serum IgG. A FACSCAN Flow Cytometer and CellQuest Software (Becton Dickinson) were used to acquire and analyze data.

PCR primers and Reverse transcription-PCR (RT-PCR). The thymus was removed from *neu/neu* mice at gestational day 18, age 7 days, age 10 weeks, or age 15 weeks (one week after weaning from pups) and the thymocytes separated from the thymic stroma using a cell strainer. RNA was then isolated using RNAzol and reverse transcription was performed using DNAse I, RNAse Inhibitor and AMV Reverse Transcriptase (Life Technologies) according to the manufacturer's specifications. The 5'- and 3'- Her-2/neu specific primers, 5'-ATTCATCATTGCAACTGTAGA-3' and 5'-AAGCACCTTCACCCTTCCTTA-3', respectively, amplify a 222bp region between bases #2140 and #2362 of the rat HER-2/neu cDNA. Primers that amplify a 260 bp fragment of β-2 microglobulin (45) were used as standard and positive control in these assays.

Whole-cell Vaccinations. On the day of vaccination, cells grown *in vitro* were trypsinized, washed three times in HBSS (pH 7.4), and counted. The cells were resuspended in HBSS at 10^7 cells/ml, irradiated with 5,000 rad from a ^{137}Cs source discharging ~1400 rad/min.

Eight-week-old *neu/neu* mice or FVB/N were given three 100 µl subcutaneous injections (right and left forelimb, left hind limb) using a 1-ml tuberculin syringe with a 27-gauge needle.

Recombinant Vaccinia Virus Vaccinations. To generate neu-N recombinant vaccinia (neu rVV), the 5 kb Hin dIII/Sal I fragment from pSV2-neu-N was cloned into pSC11-1 and recombinant vaccinia virus prepared and amplified as described (46). HER-2/neu expression was verified by FACS analysis of neu rVV-infected 3T3 cells. Eight-week-old *neu/neu* mice received four weekly injections of $3x10^7$ pfu/animal given i.p. using a 1-ml tuberculin syringe with a 27-gauge needle. Recombinant vaccinia virus expressing the influenza HA protein (HA rVV), used as a negative control, were a gift from Dr. Hyam Levitsky.

Tumor Challenge. To titrate NT cell lines in vivo, 8-week-old *neu/neu* or FVB/N mice were given subcutaneous injections of NT cells in HBSS at doses ranging from $1x10^3$ cells to $5x10^5$ cells for *neu/neu* mice and $5x10^5$ to $1x10^7$ cells for FVB/N mice. Animals were then monitored for the development of palpable (> 5 mm diameter) tumors at the challenge site. For tumor challenge experiments, *neu/neu* mice were given $5x10^4$ NT cells subcutaneously in the right hind limb 14 days after receiving a whole-cell vaccine or 7 days after the final rVV vaccination.

T cell Assays. ELISPOT analysis of neu-specific IFN-γ was performed using T cells isolated from *neu/neu* mouse spleens. Transgenic mice were given a subcutaneous NT challenge followed three days later by vaccination with 3T3-neu/GM or 3T3/GM. On day 12 post-vaccine, splenocytes were isolated by Ficoll separation and passed through a nylon wool column to remove B cell and macrophage contaminants. Neu-specific IFN-γ production was determined by a standard ELISPOT protocol (46) using $5x10^5$ NT target cells per well and serial dilutions of *neu/neu* T cells from $1x10^5$ cells per well to $1x10^3$ cell per well. Reagents used in the assay were

the following: rat anti-mouse IFN-γ at 10 μg/ml (Pharmingen), biotinylated rat anti-mouse IFN-γ at 2 μg/ml (Pharmingen), avidin-alkaline phosphatase at 2 μg/ml (Sigma), and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (Sigma).

Her-2/neu specific lysis and CD8-blockable lytic activity were determined by standard procedures (46) using splenocytes obtained 2 weeks after vaccination. Briefly, spleens were removed and splenocytes isolated by Ficoll separation. The splenocytes were incubated 5 days in the presence of murine IL-2 and IFN-γ - and mitomycin-treated NT cells. Lytic function was then determined in a 4-hour chromium-51 release assay using 3T3 and 3T3-neu cells as targets. Blocking of CD8-dependent lysis was accomplished by adding 2.43 antibody, which blocks CD8, during the 4-hour incubation. Neu-specific lysis was determined by subtracting background lysis of 3T3 cells from the lysis obtained against 3T3-neu targets.

T cell Depletion. The depletion of $CD4^+$ and $CD8^+$ T cell subsets was accomplished by giving a series of three i.p. injections of 500 μg GK1.5 (anti-CD4) or 2.43 (anti-CD8) antibody, respectively. Depletion of $CD4^+$ and $CD8^+$ T cells was verified by FACS analysis and maintained by continuing antibody injections twice weekly for the duration of the tumor challenge experiment. Anti-NK cell antibody (PK 136) was used as a negative control.

Prevention of Spontaneous Tumor Formation. Parous *neu/neu* females (18 weeks of age, 3 weeks after weaning from pups) were given a series of 5 weekly injection of $1x10^7$ pfu neu rVV (vaccine group) or no treatment (control group) and were monitored for the development of spontaneous mammary tumors.

Statistical Analyses. Statistical analyses were performed using the Statview software program. Kaplan-Meier non-parametric regression analyses for tumor-protection and tumor-

prevention experiments were performed and significance determined using the Mantel-Cox log-rank test.

RESULTS

Establishment of HER-2/neu-expressing mammary tumor cell lines. HER-2/neu transgenic mice (*neu/neu* mice) spontaneously develop focal mammary adenocarcinomas beginning at approximately 25 weeks of age (33). By 32 weeks of age, approximately 50% of transgenic females will have at least one mammary tumor. In order to facilitate the initial evaluation of neu-specific vaccine strategies for spontaneous tumor formation, five spontaneous tumors were excised from transgenic mice, digested with dispase and collagenase, and established *in vitro* using our defined breast media as described in Materials and Methods. Tumor cells were separated from untransformed epithelial cells by differential trypsinization, and the resulting HER-2/neu-expressing tumor cell lines were given the designation NT. FACS analysis of the NT lines showed significant surface HER-2/neu and MHC Class I (Fig. 1). NT cells were then used as transplantable tumors in subsequent tumor challenge experiments.

Spontaneous tumors are highly immunogenic in parental, but not transgenic mice. To evaluate the immunogenicity of the neu tumors *in vivo*, naïve FVB/N and *neu/neu* mice were given a subcutaneous tumor challenge consisting of NT cells in HBSS and monitored for the development of palpable tumors (>5 mm mean diameter). FVB/N mice were able to reject an NT challenge at doses below $5x10^6$ cells/animals (Fig. 2). In contrast, *neu/neu* mice demonstrated significant tolerance to the HER-2/neu-expressing tumor lines; the greatest dose required for

tumor growth in 100% of animals was at least 100 fold lower for the transgenic mice compared to FVB/N mice.

HER-2/neu is not expressed in the thymus of *neu/neu* mice during T cell selection. Because the transgenic mice showed significant tolerance to the neu tumors, we wanted to assess the probability that all neu-specific T cells are deleted in the *neu/neu* mice during development. We therefore examined transgene expression in the thymus of fetal and adult *neu/neu* mice by RT-PCR. Samples of mRNA were obtained from both the thymic stroma and thymic epithelium and primers were used to amplify a 300 bp fragment from the rat neu-N cDNA, using primers for β2-microglobulin as a control. As shown in Fig. 3, the data indicated that, while neu expression is seen in parous adult thymi, no such expression was detected in samples from fetal or virgin adult mice. The low-level neu expression found in newborn mice may have resulted from hormones in their milk that activated the MMTV promoter driving the rat neu-N transgene. This expression, however, is seen after the peak of T cell development, which occurs around gestational day 18. Control samples taken from parental FVB/N animals showed no detectable HER-2/neu expression.

Transgenic mice demonstrate HER-2/neu-specific immune responses. Vaccination of FVB/N mice with an irradiated whole-cell vaccine consisting of NT cells transduced to secrete murine GM-CSF results in the induction of a HER-2/neu-specific antibody response (Fig. 4) as well as the generation of HER-2/neu-specific CTL[4]. Because the transgenic mice demonstrated profound tolerance to HER-2/neu-expressing tumors *in vivo*, we sought to determine whether or not a HER-2/neu-specific immune response analogous to that observed in the parental animals could be induced by vaccination. *neu/neu* mice were vaccinated with either HBSS, irradiated NT cells, or irradiated NT/GM cells, and serum samples were obtained 14 days after vaccination.

Levels of HER-2/neu-specific serum IgG were determined by FACS analysis of mouse serum using 3T3 cells as negative targets, 3T3-neu cells as positive targets, and a FITC-conjugated pan murine IgG secondary antibody to detect the presence of IgG bound to these cells. Transgenic mice have low basal levels of HER-2/neu specific IgG (Fig. 4) which do not increase appreciably with an irradiated NT vaccine. However, vaccination with a GM-secreting NT vaccine resulted in an increase in neu-specific IgG relative to unvaccinated animals, though the level of induction was much less than that seen in FVB/N mice.

Having demonstrated the induction of neu-specific antibody responses in transgenic mice, we then sought to determine whether or not neu-specific T cell responses could also be induced in these mice. In one analysis, transgenic mice were given a subcutaneous tumor challenge ($5x10^4$ NT cells) followed three days later with either a 3T3-neu/GM vaccine (vaccine group) or a 3T3/GM vaccine (ctrl group). On day 12 post-vaccine, splenocytes were isolated, passed through a nylon wool column to remove B cell and macrophage contaminants, and assayed for neu-specific IFN-γ production by ELISPOT assay. As shown in Fig. 5a, mice receiving a neu-specific vaccine showed a significant increase in the number of T cells producing IFN-γ in response to NT cells. Surprisingly, these same T cells were unable to lyse NT cells in a 4-hour chromium-51-release assay (data not shown). Further evidence of neu-specific effector function was demonstrated by chromium-51-release assay using 3T3-neu cell targets. Animals were vaccinated with $1x10^6$ irradiated 3T3 or 3T3-neu/GM cells and splenic T cells were isolated 14 days later. T cells, cultured for 5 days in the presence of IL-2 and mitomycin- and IFN-γ - treated NT cells, were assayed for the lysis of 3T3 or 3T3-neu target cells in a 4-hour chromium-release assay. As shown in Fig. 5b, the total neu-specific lysis obtained using splenocytes from vaccinated *neu/neu* mice is much greater than that of splenocytes from unvaccinated controls.

Further, neu-specific lysis is almost completely abrogated by the addition of the CD 8-blocking antibody, 2.43.

HER-2/neu can serve as a tumor rejection target in vaccinated transgenic mice. Because we were able to demonstrate an inducible neu-specific T cell response in the form of antibody production and CTL generation, we next sought to determine whether a protective in vivo anti-tumor response could be detected despite tolerance to HER-2/neu in the transgenic mice. Animals received a vaccine consisting of three subcutaneous injections of γ-irradiated NT/GM cells given at three spatially distinct sites, totaling a dose of $3x10^6$ cells. After 14 days, the animals were given a subcutaneous tumor challenge consisting of $5x10^4$ NT cells and monitored for the growth of palpable tumors. The data demonstrate that animals given an NT/GM vaccine have a significantly improved tumor-free survival of transgenic animals relative to mock-vaccinated animals ($p < 0.0001$) (Fig. 6a) or animals receiving an irradiated NT control vaccine ($p < 0.003$) (data not shown). In a companion study, FVB/N animals given an NT/GM vaccine did not develop tumors even after a 100-fold greater NT challenge ($5x10^6$ NT cells per animals) (Fig. 6b).

To verify that HER-2/neu, specifically, was the tumor rejection antigen in the tumor challenge experiment described above, we employed two vaccination methods in which HER-2/neu was the only target antigen, whole-cell (3T3-neu/GM) and recombinant vaccinia virus (neu rVV), each of which expresses only the rat HER-2/neu cDNA in common with the mammary tumor. As shown in Fig. 7, *neu/neu* mice given a 3T3-neu/GM vaccine were significantly protected from NT challenge relative to control animals ($p < 0.005$). Results obtained with a neu rVV were equally impressive. Again, an antigen-specific response to vaccination is apparent, as *neu/neu* mice given a rVV expressing HER-2/neu were protected from NT challenge ($p < 0.01$).

Because each of these vaccines has only HER-2/neu expression in common with each other and with the NT line, HER-2/neu is clearly the relevant tumor rejection antigen.

Both $CD4^+$ and $CD8^+$ T cells are necessary for the induction of anti-tumor immunity. Next, we depleted *neu/neu* mice of either $CD4^+$ T cells or $CD8^+$ T cells prior to vaccination with 3T3-neu/GM in order to determine whether the protection we had observed in the above experiments was, in fact, T cell mediated. Animals were given i.p. injections of either the anti-CD4 GK1.5 or the anti-CD8 antibody 2.43 to deplete the respective T cell subset. Depletion was verified by FACS analysis of splenic T cells prior to vaccination. Two weeks after vaccination with 3T3-neu/GM cells, animals were given an NT challenge and monitored for the development of palpable tumors. The data, summarized in Table I, show that mice depleted of $CD4^+$ T cells develop tumors with kinetics that are similar to that seen in unvaccinated, undepleted controls. The deletion of $CD8^+$ T cells has a less dramatic effect on tumor growth, though NT growth is statistically distinct from vaccinated, undepleted animals, and is very similar to unvaccinated, undepleted controls. The tumor-free survival of animals given the NK cell-depleting antibody PK 136 and vaccine were identical to that of undepleted, vaccinated mice (data not shown). These data establish the importance of both $CD4^+$ and $CD8^+$ T cells in inducing neu-specific immunity.

Neu-specific vaccination of parous mice results in delayed spontaneous tumor growth. Because the neu-specific vaccines demonstrated efficacy in protection experiments, we sought to evaluate their effectiveness in the prevention of spontaneous tumor formation in *neu/neu* mice. After their first litter was weaned, transgenic females were given 5 weekly injections consisting of $3x10^7$ pfu neu rVV (beginning at approximately 18 weeks of age). The animals were then monitored for the development of spontaneous tumors. As shown in Fig. 8,

there was a significant delay in the onset of tumor formation in the vaccinated mice by 7 weeks, relative to unvaccinated controls ($p = 0.02$). Nearly identical results were obtained using neu-N-encoding plasmid DNA vaccination as well as whole-cell vaccination (3T3-neu/GM) (data not shown), demonstrating that the neu-specific vaccines can delay tumor onset, and suggesting that more complete protection may be possible.

DISCUSSION

Here we describe the immunological characterization of the HER-2/neu transgenic mouse model of mammary cancer developed by Muller (33). These mice demonstrate tolerance to neu relative to parental animals. However, vaccination using various neu-specific approaches can induce neu-specific T cell responses potent enough to significantly delay the development of transplantable neu-expressing tumors. Remarkably, these vaccine strategies are also potent enough to induce a significant delay in the spontaneous development of tumors. These findings demonstrate that HER-2/neu can serve as a tumor rejection target *in vivo* in this model and, further, that it should be possible to use this model to develop more potent vaccine approaches for the treatment and prevention of neu-expressing tumors.

It is clear from our data that, in the absence of tolerance, i.e. in the parental FVB/N mice, neu tumors are highly immunogenic. This is consistent with the findings of Bernards and coworkers (47), who demonstrated *in vivo* protection from HER-2/neu-transformed 3T3 cells in NFS mice after neu rVV vaccination. More recently, Chen and coworkers (48) showed that, after plasmid DNA vaccination, non-transgenic FVB/N mice were protected from a tumor challenge using Tg1-1 tumors cells, which were generated from a spontaneous mammary tumor

that arose in a transgenic mouse expressing the activating, mutant HER-2/neu protein (neu-T). Thus, our data confirm that neu is a relevant tumor rejection target in non-transgenic immunocompetent mice. However, our data also provide the first evidence demonstrating that neu is a relevant tumor rejection target in tolerized transgenic mice.

The presence of tolerance to a tumor antigen *in vivo* represents a significant challenge to successful immunotherapy of human cancers. For example, patients with breast cancer show neu-specific responses in the form of antibody and CTL, but these responses are not sufficient to prevent tumor progression (34, 35, 40). We have made a similar observation in the *neu/neu mice*. Both neu-specific CTL and neu-specific antibody responses are inducible in the transgenic and in the parental FVB/N mice. However, the immunologic responses observed in the transgenic mice are not as protective against tumor development in the transgenic mice when compared with parental mice. ELISPOT analysis of T cells isolated from transgenic mice post-vaccination demonstrate the neu-specific vaccine-dependent induction of T cells that produce IFN-γ in response to NT cells, but are unable to lyse NT cells in a chromium-51-release assay (data not shown). Despite this inability to lyse NT cells *in vitro*, CTL obtained from vaccinated *neu/neu* mice show significant lysis of neu-expressing 3T3-neu targets. Similarly, Zaks and Rosenberg (49) recently demonstrated that neu-specific CTL isolated post-vaccination from patients with neu-positive breast, ovarian, or colorectal adenocarcenomas, while capable of lysing target cells pulsed with neu-peptide, failed to react with neu-positive tumor lines. Furthermore, naive *neu/neu* mice have detectable levels of neu-specific serum IgG, which increase upon vaccination with a GM-secreting tumor cell vaccine, but not to the high levels seen in parental animals. Our data therefore provide evidence that tolerance to neu is ongoing in the HER-2/neu transgenic mice. The data also suggests that tolerance *in vivo* proceeds via

mechanisms of peripheral tolerance since HER-2/neu expression was not demonstrated in the thymus during T cell development. However, HER-2/neu expression is demonstrated in the thymus at times when the mice have higher levels of peripheral neu expression (i.e. when prolactin levels, an inducer of the MMTV promoter, are high during nursing or pregnancy). Therefore, it is probable that higher-avidity T cells that are present in the FVB/N mice and are responsible for the tumor immunogenicity observed, have undergone deletion in the transgenic mice (50). The fact that antibody and T cell responses are induced in *neu/neu* mice after vaccination but are not fully protective strongly suggests that the T cells present in *neu/neu* mice may well be of lower-avidity and have more stringent requirements for activation and effector function. It is also possible that the lower-avidity T cells undergo peripheral anergy induction in the transgenic mice.

We have shown that it is possible to significantly delay tumor growth in transgenic mice with a whole-cell vaccine derived from NT cells that produce murine GM-CSF. The fact that FVB/N mice are fully protected from a 100-fold greater tumor burden after an identical vaccination is indicative of the profound tolerance to HER-2/neu in the *neu/neu* mice. The fact that neu-specific responses are inducible at all, however, is significant and points to the potential for protective immunotherapy. Because the NT cell line may express other, as yet unidentified, tumor rejection antigens in addition to HER-2/neu, we developed separate vaccine strategies that targeted only HER-2/neu. The ability of antigen-specific vaccines (3T3-neu/GM and neu rVV) to generate anti-tumor immunity clearly implicates HER-2/neu as the *in vivo* rejection antigen. Though protection from tumor challenge was not complete in these experiments, the delay in tumor growth was statistically significant. Depletion experiments carried out in *neu/neu* mice further demonstrated that the protective effects seen in the tumor challenge experiments were, in

fact, T cell-mediated. The deletion of $CD4^+$ T cells prior to vaccination results in tumor growth that is indistinguishable from that of undepleted, unvaccinated control animals. This effect is somewhat different than what is seen in parental FVB/N mice, however. In the absence of tolerance, $CD4^+$ T cell depletion in FVB/N mice results in more rapid tumor growth than what is observed in undepleted, unvaccinated controls[4]. The difference in tumor growth kinetics in $CD4^+$ T cell-depleted FVB/N mice relative to control animals probably results from the loss of spontaneous neu-specific antibody generation, which is seen even in unvaccinated mice after tumor challenge. This would be consistent with previous findings demonstrating that treatment of neu-expressing cell lines with antibodies against HER-2/neu results in growth arrest and apoptosis of the neu-positive cell lines (51). It is unlikely that $CD4^+$ T cells play a direct role in tumor cell lysis, as even after IFN-γ treatment, NT cells express little or no MHC Class II. Because NK depletion has no effect on the tumor-free survival of vaccinated animals, it seems unlikely the $CD4^+$ T cell-mediated NK-dependent lysis plays an important role in NT rejection. Thus, the role of $CD4^+$ T cells in tumor rejection appears to be at the level of T cell help for both cellular and humoral responses. In contrast to the effects of $CD4^+$ T cell depletion, the depletion of $CD8^+$ T cells has a less dramatic effect on tumor protection in *neu/neu* mice. $CD8^+$ T cell-depleted animals demonstrate a significant delay in tumor growth relative to undepleted, unvaccinated controls despite the loss of neu-specific CTL. The statistical difference in tumor free survival between the $CD8^+$ T cell-depleted group and the control group ($p = 0.02$) is most likely due to antibody-dependent growth inhibition, as the $CD8^+$ T cell-depleted mice are still capable of antibody induction after vaccination. The delayed tumor growth observed in $CD8^+$ T cell depleted mice is not as great as that seen in the undepleted, vaccinated mice relative to controls ($p = 0.007$), which likely reflects the fact that the undepleted, vaccinated mice are

capable of both antibody production and CTL induction. The effect of $CD8^+$ T cell depletion seen in FVB/N mice is much greater than that seen in the *neu/neu* mice[4], which further suggests that higher-avidity T cells have been deleted in the transgenic mice. Taken together, the $CD4^+$ and $CD8^+$ T cell depletion experiments presented here suggest that in the neu-N transgenic model, the induction of an antibody response to vaccination is important for the retardation of tumor growth, as demonstrated by the protection observed in $CD8^+$ T cell-depleted animals. However, a more efficient $CD8^+$ T cell response to tumor *in vivo* is necessary for complete protection.

Our studies also show that it is possible to induce protective immunity capable of delaying the development of spontaneous mammary tumors in the transgenic mice. When mice were given a series of neu-specific vaccinations, it was possible to delay the onset of spontaneous tumor growth in post-lactational *neu/neu* mice by 49 days, relative to controls. The prevention of spontaneous tumor formation has been reported in the neu-T transgenic mouse model using i.p. injections of antibody against HER-2/neu (52) as well as plasmid DNA immunization (53), demonstrating the successful use of immunotherapy for tumor prevention. However, tumor incidence in the neu-T transgenic mouse model for breast cancer occurs in a single-step fashion (54, 55), suggesting that neu-T overexpression alone is sufficient for oncogenic transformation (43, 56). In the neu-N transgenic mouse model used in these studies, tumor incidence occurs in a stochastic manner indicating that neu-N overexpression is necessary for transformation, but that other steps are required before the cells take on the transformed phenotype (43, 56). For this reason, the neu-N model is a more clinically relevant system in which to evaluate anti-tumor vaccine approaches. The fact that we were able to delay tumor onset in these mice is significant in that it demonstrates that we can generate a neu-specific

response in HER-2/neu transgenic mice that is capable of overcoming *in vivo* tolerance and is sufficient to prevent spontaneous tumor development. Boggio and coworkers were able to prevent spontaneous tumor developments in both neu-N and neu-T transgenic mouse models with i.p. injections of IL-12 given every fourth week (57). In neu-N mice, 50% of the mice were tumor free at 61 weeks of age in the treatment group, however the mechanism of protection is believed to be related to the downstream anti-angiogenic effects associated with IL-12 treatment and not through a T cell-dependent mechanism (57). Clearly, further work is required to augment the vaccine in such a way that the tumor protective effects can be maintained indefinitely. However, these studies suggest that it may also be possible to target early events involved in tumor development to prevent cancer. It may be possible to maintain the tumor preventive effects reported here by boosting with multiple vaccinations in post-lactational females. We are also investigating the possibility that the induction of high titers of neu-specific IgG can synergize with T cell effector function in the eradication of neu tumors. The modification of neu-specific vaccines to enhance both cellular and humoral responses (neu-specific antibody production) may be another effective method to boost the protective effects we have demonstrated here. In addition, the use of dendritic cell vaccines may facilitate more effective T cell priming, thereby generating a more potent immune response *in vivo*. Finally, it may be possible to amplify the lower-avidity neu-specific T cells by including both costimulatory signals (B7-1, etc.) and T cell-activating signals (CD40 ligand, etc) to our current vaccine approaches.

In summary, we have demonstrated the feasibility of using the neu-N transgenic mouse model of mammary cancer to study immune-activating anti-cancer approaches. This model should prove useful for studying anti-tumor immune responses in a natural tumor model that has

greater relevance to human cancer than previously described pre-clinical models. Vaccine approaches that are shown to be effective in this model may result in more potent immune approaches in patients with cancer.

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FIGURE LEGENDS

Fig. 1. Transplantable mammary tumor lines express HER-2/neu and MHC Class I. Tumor cells, derived from spontaneous mammary tumors in transgenic mice and established *in vitro*, were analyzed by FACS for surface protein expression using primary antibodies against HER-2/neu (c-neu Ab4) and MHC Class I (28-14-8s). (*A*) NT cell Line, early passage. (*B*) NT cells explanted from *neu/neu* mouse after tumor challenge. (*C*) NT cell line, after > 25 passages. Levels of HER-2/neu (dotted line) and MHC I (gray line) show a significant shift in fluorescence relative to the curve obtained using an irrelevant antibody (W632, black line).

Fig. 2. Neu tumor cells are immunogenic in parental but not transgenic mice. FVB/N (open symbols) and *neu/neu* mice (closed symbols) were given a subcutaneous tumor challenge in the right hind limb that consisted of the indicated number of NT cells suspended in HBSS. Animals were then monitored for the development of palpable tumors.

Fig. 3. HER-2/neu expression is not detected in the thymus during the peak of T cell development. The thymic stroma and thymocytes were obtained at gestational day 18 (fetal), age 7 days (fetal), age 10 weeks (adult) and post-lactational (parous) *neu/neu* mice and mRNA was extracted. RT-PCR was then performed with primers for HER-2/neu, using primers for β-2 microglobulin as a positive control. HER-2/neu expression was detected in mRNA samples from a neu-expressing cell line (NT Line) as well as in the thymic stroma of parous females and in both the stroma and thymocytes of newborn mice. No neu signal was detected in thymic stroma from non-transgenic mice (FVB/N S.).

Fig. 4. A neu-specific whole-cell vaccine elicits an antibody response in both FVB/N and transgenic mice. Serum was obtained from FVB/N and *neu/neu* mice 14 days after vaccination and used as the primary antibody solution in a FACS analysis of both 3T3 and 3T3-neu cells using a secondary antibody against murine IgG. The mean fluorescence intensity shown is the difference between that obtained for the staining of 3T3 and that obtained for the staining of 3T3-neu cells after vaccination with Hanks Balanced Salt Solution, NT cells or NT/GM cells.

Fig. 5. Neu-specific vaccination of transgenic mice results in an increase in neu-specific T cells. (*A*) ELISpot analysis was used to determine the number of neu-specific T cells induced *neu/neu* mice with a 3T3-neu/GM vaccine. Transgenic mice were given a subcutaneous NT challenge followed three days later by vaccination with 3T3-neu/GM or 3T3/GM. Spleens were removed 12 days later and splenocytes obtained by Ficoll separation. ELISpot analysis was then used to detect the production of IFN-γ in response to NT cells. We observed a three- to four-fold increase in neu-specific T cells after vaccination with 3T3-neu/GM relative to mock-vaccinated controls. (*B*) A 4-hour chromium-release assay was performed using splenocytes isolated from transgenic mice to determine the relative increase in neu-specific lysis of 3T3-neu cells after vaccination. Splenocytes were isolated from *neu/neu* mice 14 days after vaccination with 3T3-neu/GM, incubated 5 days in the presence of mitomycin-treated and IFN-γ-treated NT cells, and used in a 4-hour chromium-release assay. Both 3T3 and 3T3-neu cells were used as targets in the assay. The neu-specific lysis shown in the graph for vaccinated (open squares) and unvaccinated (open triangles) groups was calculated by subtracting the percent lysis against 3T3

cells from that obtained using 3T3-neu cells. The open circles represent neu-specific lysis in the presence of the CD8-blocking antibody 2.43.

Fig. 6. Transgenic mice are protected from a neu tumor challenge after NT/GM vaccination. (*A*)Transgenic mice were vaccinated with $1x10^6$ NT/GM cells at each of 3 subcutaneous sites. Two weeks after vaccination, the mice received a subcutaneous NT challenge ($5x10^4$ cells) in the right hind limb and monitored for the presence of palpable tumor. Vaccinated mice (open squares) (n = 45) showed a significant delay in tumor growth relative to mock-vaccinated controls (filled diamonds) (n = 41) ($p < 0.0001$). (*B*) FVB/N mice (n = 8) given an NT/GM vaccination (open circles) did not develop tumor after NT challenge with $5x10^6$ cells. Mock-vaccinated animals were used as a control group (filled diamonds).

Fig. 7. HER-2/neu is the *in vivo* rejection antigen in transgenic mice. Transgenic mice received either a 3T3-neu/GM vaccine (open squares) (n = 10) or neu recombinant vaccinia virus (open triangles) (n = 10) followed by a subcutaneous NT challenge in the right hind limb. Animals were then monitored for the formation of palpable tumors. Animals receiving a neu-specific vaccine were protected from NT challenge relative to the corresponding control animals (3T3 – filled diamonds, HA rVV – filled circles) with p values of 0.005 and 0.01 for the whole-cell and rVV groups, respectively.

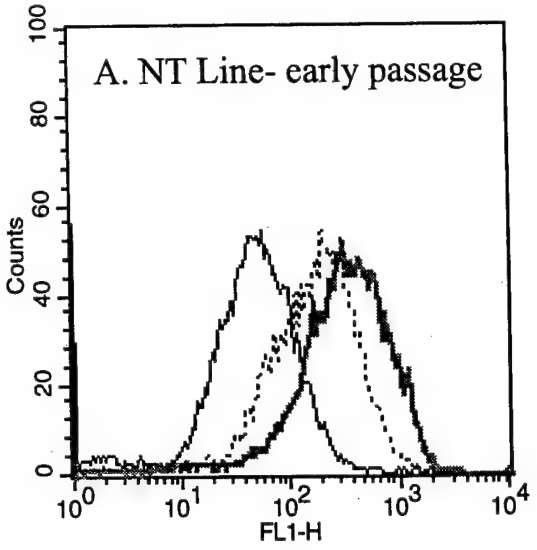
Fig. 8. Neu-specific vaccination is protective against spontaneous tumor formation in transgenic mice. Spontaneous tumors begin to arise at approximately 22 weeks of age in post-lactational *neu/neu* mice. A series of 5 neu rVV vaccinations were given to *neu/neu* mice

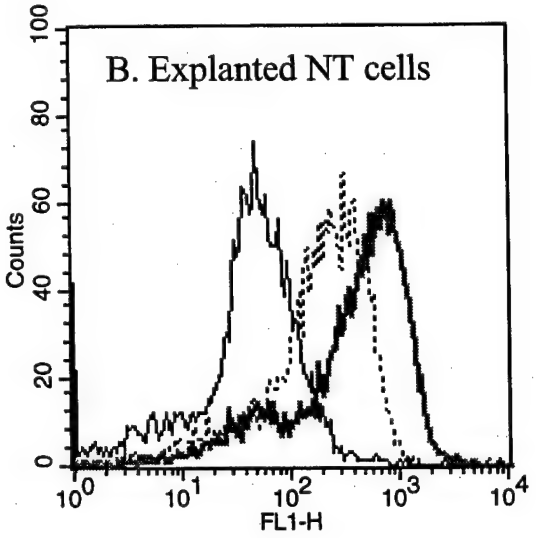
beginning at 18 weeks of age (3 weeks after weaning from pups) and the mice monitored for spontaneous mammary tumor formation. Vaccinated animals (open squares) (n = 12) showed a significant delay in the onset of tumor formation relative to age-matched unvaccinated post-lactational control animals (filled diamonds) (n = 10) ($p = 0.02$ at day 84). Similar results were obtained with whole-cell vaccination and plasmid DNA vaccination (data not shown).

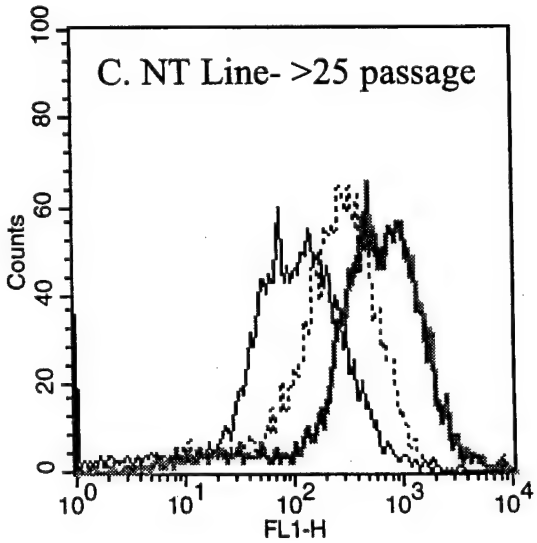
Table I. Effects of T cell Depletion in *neu/neu* Mice

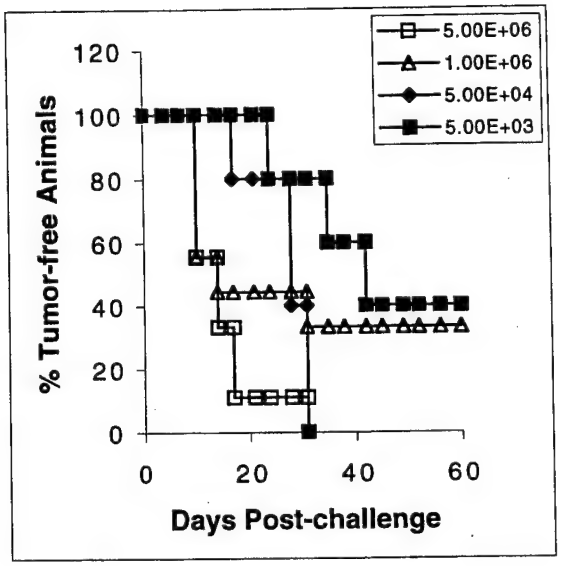
Group	% Tumor-Free (day 24)	p value
no dep, no vac	0	---
no dep, vac	75	0.007 (vs. no vac)
CD4 dep, vac	0	0.31 (vs. no vac)
CD8 dep, vac	40	0.02 (vs. no vac)
NK dep, vac	68	0.004 (vs. no vac)

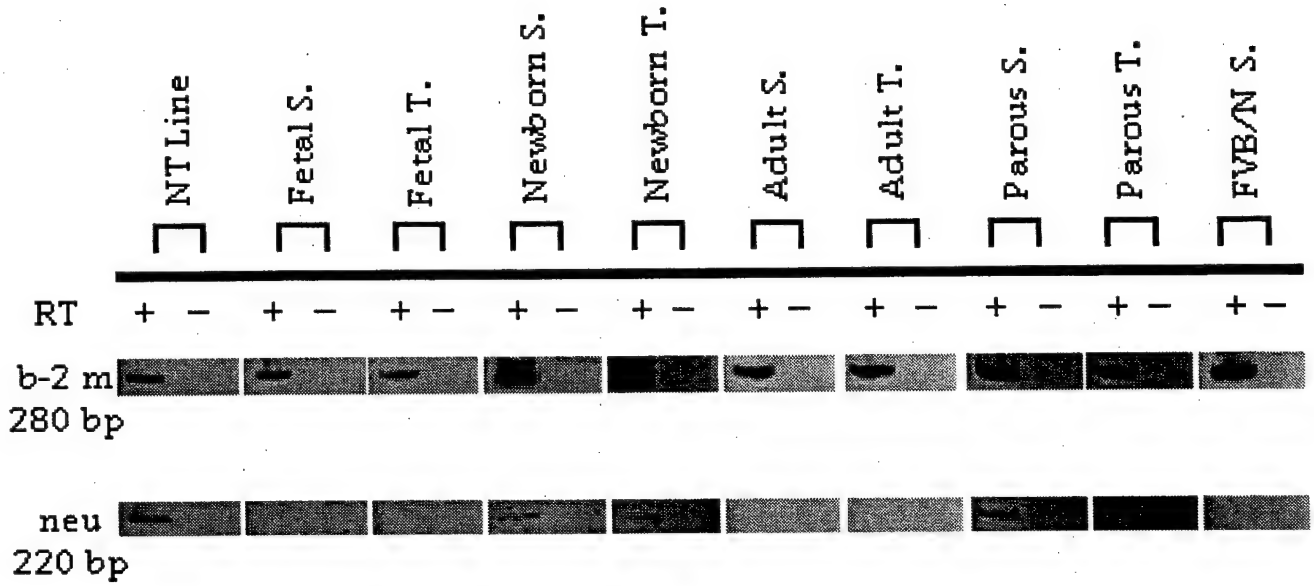
Transgenic mice were depleted of T cell subsets and given a challenge consisting of $5x10^4$ NT cells. Protection from s.c. injection of NT (% tumor-free) is given at day 24 post-challenge, at which time all control animals had developed palpable tumor. Control animals (no dep, no vac) received HA rVV, all other groups received neu rVV. Statistical significance values were determined using the Logrank test as compared to the control group.

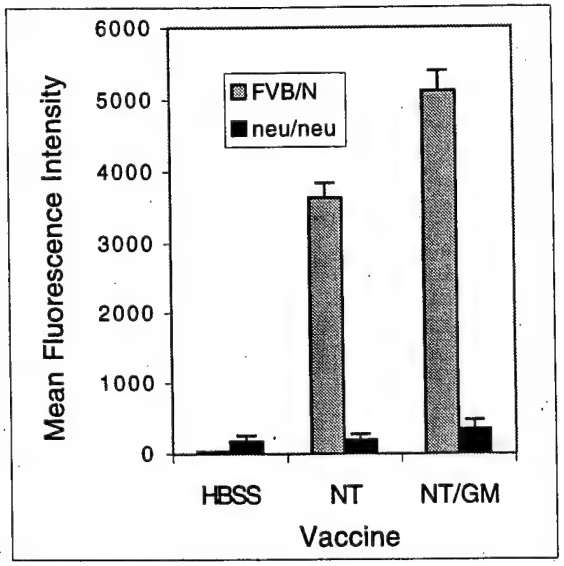

A. NT Line- early passage
Counts
FL1-H

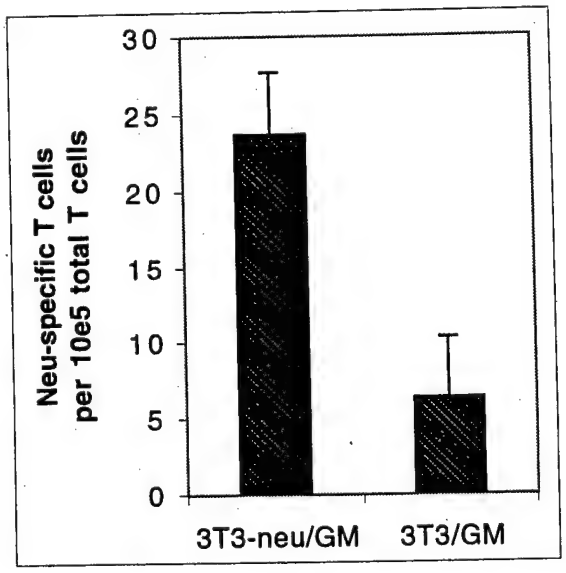

B. Explanted NT cells
Counts
FL1-H

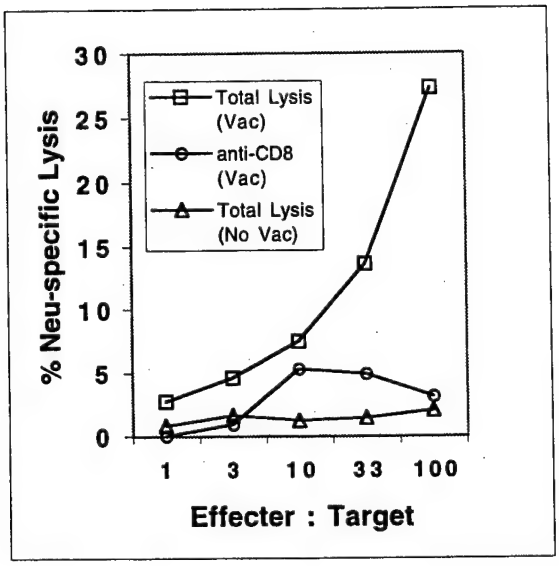

C. NT Line- >25 passage
Counts
FL1-H

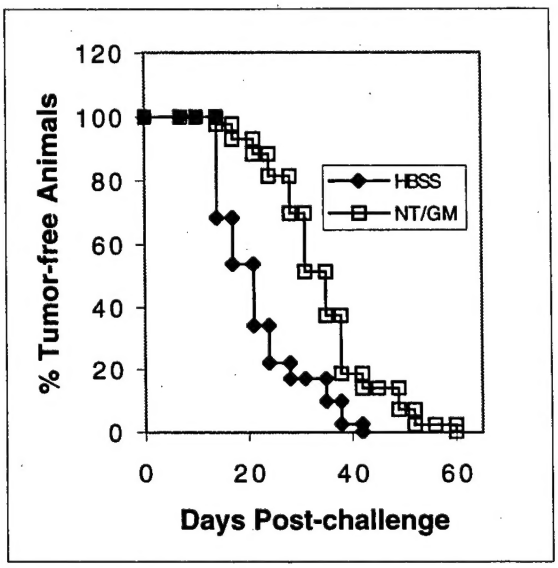

5.00E+06
1.00E+06
5.00E+04
5.00E+03
% Tumor-free Animals
120
100
80
60
40
20
0
0
20
40
60
Days Post-challenge

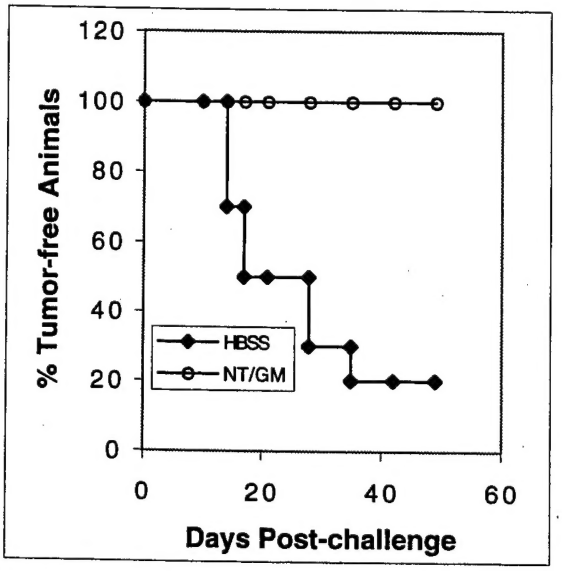

NT Line
Fetal S.
Fetal T.
Newborn S.
Newborn T.
Adult S.
Adult T.
Parous S.
Parous T.
FVB/N S.
RT + − + − + − + − + − + − + − + − + − + −
b-2 m
280 bp
neu
220 bp

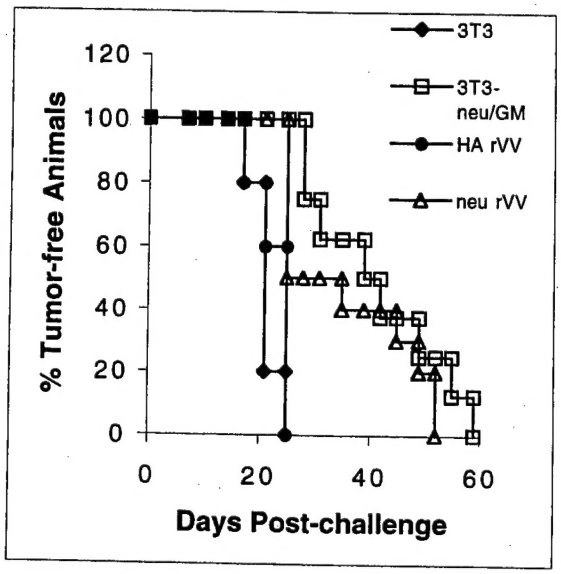

6000
5000
4000
3000
2000
1000
0
Mean Fluorescence Intensity
FVB/N
neu/neu
HBSS
NT
NT/GM
Vaccine

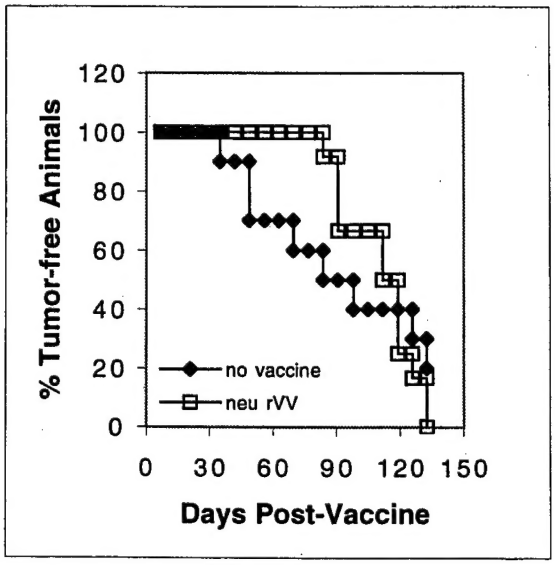

Neu-specific T cells
per 10e5 total T cells
30
25
20
15
10
5
0
3T3-neu/GM
3T3/GM


30
25
20
15
10
5
0
% Neu-specific Lysis
Total Lysis (Vac)
anti-CD8 (Vac)
Total Lysis (No Vac)
1
3
10
33
100
Effecter : Target


120
100
80
60
40
20
0
% Tumor-free Animals
0
20
40
60
Days Post-challenge
HBSS
NT/GM


120
100
80
60
40
20
0
% Tumor-free Animals
HBSS
NT/GM
0
20
40
60
Days Post-challenge


3T3
3T3-neu/GM
HA rVV
neu rVV
% Tumor-free Animals
120
100
80
60
40
20
0
0
20
40
60
Days Post-challenge


% Tumor-free Animals
120
100
80
60
40
20
0
no vaccine
neu rVV
0
30
60
90
120
150
Days Post-Vaccine